The Role of Fibroblasts in Cancer Progression, Therapy Resistance and Field Cancerization

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List of abbreviations

5-FU	5-fluorouracil
AK	actinic keratosis
AF	activation function
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
α -SMA	alpha smooth muscle actin
Bcl-2 family	B-cell lymphoma protein family
BrdU	bromodeoxyuridine
CCM	CAF conditioned medium
CREB	cAMP response element binding protein
CSC	cancer stem cell
CAF	cancer-associated fibroblast
CCL5	chemokine (C-C motif) ligand 5
CBL	cisplatin + bicalutamide + LE135 treatment
CB	cisplatin + bicalutamide treatment
CL	cisplatin + LE135 treatment
CV	cisplatin + vehicle treatment
JNK	c-jun N-terminal kinase
CM	conditioned medium
CK	cytokeratin
DNA	deoxyribonucleic acid
DCF	dichlorofluorescein diacetate
DBD	DNA binding domain
DNMT1	DNA methyltransferase
DMEM	Dulbecco's Modified Eagle's Medium
EndMT	endothelial-mesenchymal transition
ES	enrichment score
EGFR	epithelial growth factor receptor
EMT	epithelial-mesenchymal transition
EDTA	ethylenediaminetetraacetic acid
ECM	extracellular matrix
FAP	fibroblast activation protein
FGF	fibroblast growth factor
FSP1	fibroblast specific protein 1
FACS	fluorescence associated cell sorting
FFPE	formalin-fixed paraffin-embedded
GO	gene ontology
geNORM	geometric averaging of multiple internal control genes
GLUT1	glucose transporter 1
HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor

HPLC	high-performance liquid chromatography
HDAC3	histone deacetylase 3
H ₂ O ₂	hydrogen peroxide
IPA	Ingenuity Pathway Analysis
IGF	insulin growth factor
IGFBP	insulin growth factor binding protein
IL-6	interleukin 6
IL-8	interleukin 8
IFP	interstitial fluid pressure
Keratinocyte-SFM	keratinocyte serum-free medium
LDH-B	lactate dehydrogenase B
LCM	laser capture microdissection
LBD	ligand binding domain
LEF/TCF	lymphoid enhancer factor/T-cell factor
mTOR	mammalian target of rapamycin
MMP	matrix metalloproteinase
MFAP5	microfibrillar associated protein 5
MAPK/Erk	mitogen activated protein kinase/extracellular signal-regulated kinase
MCT1/MCT4	monocarboxylate transporter 1 or 4
MCP-1	monocyte chemoattractant protein 1
NAC	N-acetyl cysteine
NK cell	natural killer cell
NADH	nicotinamide adenine dinucleotide
NSG mice	NOD scid gamma mice
NMSC	non-melanoma skin cancer
FIB	normal fibroblast
CAF-NR-OE/KD	NR overexpressing or knockdown CAFs
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NR	nuclear receptor
NCoR	nuclear receptor co-repressor
OTC	organotypic coculture
OXPHOS	oxidative phosphorylation
PTEN	phosphatase and tensin homolog
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinase
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PEG	polyethylene glycol
PCNA	proliferating cell nuclear antigen
PI	propidium iodide
Akt	protein kinase B

ROS	reactive oxygen species
RT-qPCR	real-time quantitative polymerase chain reaction
RTK	receptor tyrosine kinase
Rb	retinoblastoma protein
RNA	ribonucleic acid
Rpl27	ribosomal protein L27
SF-OTC medium	serum-free organotypic co-culture medium
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMT	somatic mutation theory
SCC	squamous cell carcinoma
TBP	TATA-box promoter
TBAB	tetrabutylammonium bromide
TAB2	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2
TIMP	tissue inhibitors of metalloproteinases
TOFT	tissue organization field theory
TGFβ	transforming growth factor-beta
TAK1	transforming growth factor-beta activated kinase
TGFβRII	transforming growth factor-beta receptor 2
TBL	trichome birefringence-like protein
p53	tumor protein p53
TAM	tumor-associated macrophage
UVA	ultraviolet radiation A
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
YAP	yes-associated protein
GSH	reduced glutathione
GSSG	oxidized glutathione
tFIB	transformed fibroblast
ELISA	enzyme-linked immunosorbent assay
SOD	superoxide dismutase
GPx1	glutathione peroxidase 1
IL1-r	interleukin 1 receptor
IL-1β	interleukin 1-beta
Nrf2	nuclear factor (erythroid-derived 2)-like 2

Authorship of publications in peer-reviewed journals

1. Angiopoietin-like 4 Mediates Colonic Inflammation by Regulating Chemokine Transcript Stability via Tristetraprolin. *Scientific Reports*; 2017 Mar 13;7:44351. doi: 10.1038/srep44351.
2. Cancer-associated fibroblasts enact field cancerization by promoting extratumoral oxidative stress. *Cell Death and Disease*; 2017 Jan 19;8(1):e2562. doi: 10.1038/cddis.2016.492.
3. Conditional knockout of N-WASP in mouse fibroblast caused keratinocyte hyper proliferation and enhanced wound closure. *Scientific Reports*; 2016 Dec 2;6:38109. doi: 10.1038/srep38109.
4. Comparative study of adipose-derived stem cells and bone marrow-derived stem cells in similar microenvironmental conditions. *Experimental Cell Research*; 2016 Nov 1;348(2):155-164. doi: 10.1016/j.yexcr.2016.09.012.
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6. Angiopoietin-like 4 stimulates STAT3-mediated iNOS expression and enhances angiogenesis to accelerate wound healing in diabetic mice. *Molecular Therapy*; 2014 Sep;22(9):1593-604. doi: 10.1038/mt.2014.102. Epub 2014 Jun 6.
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Thesis Summary

Cancer refers to a cluster of diseases that are characterized by uncontrolled neoplastic growth manifesting in a wide variety of tissues and organs. The malignant progression of cancer requires a permissive microenvironment comprising tumor-supportive cells embedded within an altered extracellular matrix (ECM), collectively known as the reactive tumor stroma. Cancer-associated fibroblasts (CAFs) are the dominant cell type within the reactive tumor stroma, contributing mitogenic factors, reactive oxygen species (ROS), metabolic intermediates, ECM molecules and matrix remodeling enzymes that profoundly influence the aggressiveness of tumor epithelia and the tumor response to therapy. Most anticancer treatments to date are targeted at the tumor epithelia and neglect the critical roles played by CAFs. Moreover, tumor resistance and relapse are persistent challenges faced by patients and clinicians, suggesting an incomplete understanding of tumor-stroma co-evolution that severely limits the sustained efficacy of existing anticancer drugs.

Herein, we explored the transcription-based programs that enable the pro-tumorigenic effects of CAFs. Firstly, we focused on the expression profile of nuclear receptors in CAFs. Because nuclear receptors are a unique class of transcription factors whose activities are directly modulated by small molecule ligands, they hold untapped potential for CAF-directed anticancer therapy. We showed that tumor epithelia are sensitive to the nuclear receptor profile of CAFs. The overexpression or knockdown of specific nuclear receptors in CAFs resulted in the attenuation of one or more aggressive phenotypes in tumor epithelial cells, namely, proliferation, invasiveness, drug resistance, energy metabolism and oxidative stress. Furthermore, we identified androgen receptor and retinoic acid receptor β antagonists as useful

adjuvants to cisplatin chemotherapy that attenuated chemoresistance in mice tumor xenografts.

Given our observations that CAFs augment oxidative stress within the tumor microenvironment, we investigated whether the readily diffusible ROS, H_2O_2 , could promote oncogenic transformation of adjacent normal epithelia and stromal fibroblasts. Indeed, epithelial cells at various stages of oncogenic transformation exhibited a differential response to H_2O_2 in terms of proliferation and invasiveness. Fibroblasts exposed to H_2O_2 also adopted a CAF-like state and became a source of H_2O_2 due to impaired TGF β signaling that resulted in a defect in ROS detoxification. Importantly, oxidatively transformed fibroblasts retained their tumor-promoting capacity when adoptively transferred into tumor xenografts.

Therefore, the findings reported in this thesis suggest that future anticancer therapies should consider the stromal context of tumors. Druggable features of CAFs such as their nuclear receptor expression and their pro-oxidant status can be targeted in combination with existing cytotoxic agents or antibody-based treatments to potentially improve patient outcomes.

Chapter 1: Introduction

1.1. Cancer as a multi-faceted disease

Cancer is the collective term for a cluster of diseases that are characterized by uncontrolled neoplastic growth manifesting in a wide variety of tissues and organs. Depending on the site in which the primary neoplastic lesion first appears, cancers are given the appropriate nomenclature to reflect their origin. For example, epithelial tumors are classified as carcinomas, blood cancers are referred to as leukemias and cancers of the connective tissue are known as sarcomas. The heterogeneity within each cancer class necessitates further sub-categorization. For example, carcinomas can be divided into squamous or basal cell carcinomas, with increasingly precise molecular subtypes branching off from both groups.

The GLOBOCAN project, which catalogs cancer morbidity and mortality globally, reports that in 2012 alone, 8.2 million people succumbed to cancer and 32.6 million people are afflicted with the disease. It projects that the number of new cancer diagnoses will increase from 14.1 million in 2012 to 22 million within 20 years. This underscores an urgent need to understand the mechanisms that initiate or permit incipient neoplasia and drive tumorigenesis. Gender, genetic susceptibility, environmental carcinogens, pathogenic infection and lifestyle factors have been shown to predispose an individual toward developing particular types of cancer. When discussing these predispositions, a dominant theme of genomic instability surfaces. The clonal origin model of cancer proposes that a cell bearing mutations that deregulate fundamental cellular processes gains a growth advantage resulting in a rapidly expanding population of abnormal clones. These hyperproliferative clones disrespect local boundaries, disrupt the structural and functional integrity of their adjacent milieu, and culminate their malignant progression by propagating to distal sites within an organism, a process called metastasis that eventually leads to the death

of the organism. Several decades of cancer research, however, have revealed that this progress towards malignancy is far more complex than this picture suggests. Over and above genomic instability, cancer cells need to coordinately acquire multiple other traits and even enlist microenvironmental support before they can thrive in a biological system that can be viewed as unaccommodating of rogue cells.

Figure 1. The hallmarks of cancer. Source: Hanahan and Weinberg's "Hallmarks of Cancer: The next generation" published in the journal, *Cell*, Volume 155, Issue 5, p646-674 in 2011. The inner circle of the schematic illustrates the 8 hallmarks and 2 enabling characteristics of cancer. The outer circle highlights the therapeutic strategies that are made possible by a deeper understanding of each individual cancer characteristic.

Hanahan and Weinberg, in their comprehensive and insightful review, identified eight hallmarks and two enabling characteristics of cancer that explain how cancer cells overcome biological barriers to achieve uncontrolled, yet sustainable

growth (Hanahan and Weinberg, 2011) (**Figure 1**). Hallmarks embody observed commonalities across cancer types whereas enabling characteristics encourage the acquisition of said hallmarks. The eight hallmarks are - potentiated proliferative signaling, insensitivity to growth suppression, resistance to apoptosis, replicative immortality, induction of angiogenesis, enhanced invasiveness and metastatic potential, metabolic reprogramming and immunoevasion. **Genomic instability** is a well-established enabling characteristic of cancer; however, in recent years, **chronic low-grade inflammation** has also been recognized as an enabling characteristic.

Potentiated proliferative signaling by cancer cells arises from a persistent availability of mitogens and their cognate receptors on cancer cells, as well as augmentation to components of their downstream signaling cascades. Cancer cells upregulate the expression of growth factors, the receptors to these factors, or both, thereby enforcing autocrine mitogenic signaling which often functions in a positive feedback loop (Perona, 2006). Alternatively, growth factors are sequestered in the extracellular matrix (ECM), and cancer cells are able to liberate and access these by degrading components of the ECM using secreted proteases (Egeblad and Werb, 2002). Another explanation for chronic proliferative signaling is directly linked to genomic instability. Stochastic mutations of genes encoding growth factor receptors and/or their downstream effectors have been shown to permit constitutive, ligand-independent downstream signaling. Mutant receptor tyrosine kinases (RTKs) with lowered thresholds to auto-phosphorylation are prone to exhibit ligand-independent activity (Lemmon and Schlessinger, 2010). Similarly, hyperactive PI3K isoforms and Ras mutants with impaired GTPase activity prolong mitogenic signaling. Individually or cumulatively, these changes result in cancer cell proliferation independent of external cues, thereby bypassing the cell growth-and-division cycle restrictions that

maintain cell number and homeostasis (Jiang and Liu, 2009; Pylayeva-Gupta et al., 2011).

Insensitivity to growth suppression is a powerful complement to the sustained proliferative signaling in cancer cells. By becoming unresponsive to growth inhibitory signals, cancer cells gain even greater autonomy from the normal cell growth-and-division cycle. One key difference observed in cell culture is the loss of contact inhibition in cancer cells. Several studies have demonstrated that cell adhesion molecules (e.g., cadherins, integrins and nestins) and membrane-cytoskeletal scaffolding proteins (e.g., Merlin) involved in contact inhibition are downregulated in cancer cells (Buda and Pignatelli, 2011; Li et al., 2012a; Takai et al., 2008). Consequently, transcription co-factors that were previously tethered by these cell adhesion and scaffolding molecules to the cytoplasmic face of the plasma membrane become free to enter the nucleus and activate pro-oncogenic transcription. For example, loss of E-cadherin has been shown to release beta-catenin from the cell periphery and increase the latter's localization to the nucleus, where it associates with the transcription factor LEF/TCF (Jamieson et al., 2014). Similarly, loss of Merlin impedes the Hippo pathway, allowing the nuclear localization of YAP, which positively regulates cell proliferation (Li et al., 2012a). In addition to contact inhibition, the most well-studied growth suppression pathways involve the retinoblastoma (Rb) and p53 proteins, both of which are transcription factors that primarily function as the colloquial “brakes” of the cell growth-and-division cycle (Bieging et al., 2014; Manning and Dyson, 2012). These proteins institute cycle arrest and even apoptosis depending on the integration of subtly different cues. Unsurprisingly, the vast majority of cancers bear inactivating mutations on genes encoding Rb and p53, rendering them insensitive to growth inhibition signals.

Resistance to apoptosis is common in cancer cells. Cancer cells do not activate the intrinsic and extrinsic apoptotic pathways even in the face of irreparable intracellular trauma (e.g., DNA damage and mitochondrial dysfunction) and/or unfavorable extracellular conditions such as nutrient deprivation and hypoxia. Traditionally, this has been attributed to (1) upregulation of anti-apoptotic Bcl-2 family protein expression, (2) downregulation of pro-apoptotic Bcl-2 family protein expression, (3) production of defective copies of pro-apoptotic Bcl-2 family members and (4) de-coupling of p53-mediated cell state curation from apoptosis triggers (Adams and Cory, 2007; Cory and Adams, 2002; Cotter, 2009). However, the evasion of apoptosis can also be perceived as an ability of cancer cells to buffer the debilitating effects of nutrient deprivation and hypoxia. Autophagy has since been implicated, and cancer cells have been shown to utilize autophagy to derive and redirect metabolites to stave off starvation. Interestingly, many stress-sensing mechanisms that trigger apoptosis also give cells the option to trigger autophagy as well (Levine and Kroemer, 2008). The PI3K/Akt/mTOR axis and the Beclin-1/Bcl-2 pathway are examples of mechanisms by which cancer cells may elect to undergo autophagy rather than programmed cell death (Mathew et al., 2007; Sinha and Levine, 2008).

Enabling replicative immortality refers to the ability of cancer cells to undergo virtually limitless rounds of mitotic division. In normal cells, a shortening of their telomeres, the protective chromosome caps, institutes an inevitable countdown towards senescence or programmed cell death (Mathon and Lloyd, 2001). Cancer cells evade this countdown by expressing the enzyme telomerase, which permits the infinite extension and repair of their telomeres (Artandi and DePinho, 2010; Novak, 2003). Paradoxically, telomere dysfunction has also been reported to promote cancer progression. This may seem counter-intuitive until one considers telomerase loss-of-

function within the context of coordinate p53 loss-of-function. Cancer cells with inactivating p53 mutations while lacking telomerase activity become particularly susceptible to chromosomal aberrations and mutation. The heightened genomic instability that ensues increases the probability of more aggressive cancer cell clones emerging (Artandi and DePinho, 2010).

Inducing angiogenesis is another defining feature of cancer and is responsible for the disease's nomenclature. Early macroscopic observations of tumors identified a "crab-like", disorganized network of vasculature extending from the neoplastic lesion. The recruitment of endothelial cells and the *de novo* establishment of blood vessels in cancer is crucial if the proliferative cancer cells are to meet their elevated respiratory and metabolic loads. While cancer angiogenesis has largely been attributed to the VEGF ligand, several cancers have been shown to also up-regulate PDGF and FGF to further potentiate the angiogenic switch (Ferrara, 2009; Heldin, 2013; Liang et al., 2013). Inhibitors of angiogenesis, or the lack of thereof, also play a role in the formation of new blood vessels in the vicinity of tumors (Lawler and Lawler, 2012). Often, these pro- and anti-angiogenic signals do not originate from the cancer cells themselves, but rather are supplied by the tumor microenvironment. The aforementioned pro-angiogenic growth factors can be found immobilized within the ECM. Matrix metalloproteinases (MMPs) produced by cancer cells then release these factors to promote angiogenesis. The various cellular populations within the tumor stroma can also contribute to blood vessel formation around tumors. Cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) are potent sources of pro-angiogenic ligands (Murdoch et al., 2008; Seton-Rogers, 2013).

Enhanced invasiveness and metastatic potential are cancer cell behaviors that stem from a fundamental phenotypic change from well-adhered, epithelial-like

cells to cells with a more mesenchymal character. The induction of a transcription-factor driven program involving Snail, Twist, Slug and Zeb has been identified as a major initiator of this phenotypic change, now widely coined the epithelial-mesenchymal transition, or EMT (De Craene and Berx, 2013; Peinado et al., 2007). Typically, a change in the subtype of cadherin expression (E-cadherin to N-cadherin) is seen in cells undergoing EMT, and cells that have undergone EMT relinquish their stable cell-cell contacts for more transient cell-ECM attachments that are well-suited for cellular migration (Peinado et al., 2007). Mesenchymal markers, such as vimentin are upregulated in transitioned cells, as is MMP secretion, which allows ECM and basal lamina degradation (Kessenbrock et al., 2010; Sutoh Yoneyama et al., 2014). Once detachment from and degradation of the basal lamina are complete, cancer cells are then free to invade rampantly and access the neovasculature surrounding the tumor. Intravasation into the bloodstream, dispersal via the systemic circulation, extravasation and establishment of secondary tumors eventually complete the process of metastasis.

Reprogramming energy metabolism is a recently recognized commonality among cancers. Unlike normal cells that derive energy in the form of ATP from the oxidative phosphorylation (OXPHOS) of glucose, cancer cells display a preference for glycolysis, termed the Warburg effect (Bensinger and Christofk, 2012; Upadhyay et al., 2013). Expectedly, cancer cells have a higher glucose uptake to compensate for the markedly lower ATP yield per molecule of glucose from glycolysis. The switch to glycolysis may be explained by the hypoxic conditions that prevail when tumor growth exceeds angiogenesis. With a limited oxygen supply, there is a selection pressure favoring cancer cells that remain able to meet their energy demands using glycolytic pathways (Hsu and Sabatini, 2008). Another proposed reason is the higher

incidence of mitophagy and mitochondrial dysfunction in cancer cells, which forces them to adapt using alternative energy-generating pathways (Wallace, 2012). There are also advantages to the glycolytic switch in cancer cells resulting from the formation of high-energy intermediates that can be channelled to anabolic processes such as protein and DNA synthesis. Even more fascinating is the concept of metabolic symbiosis between hypoxic cancer cells and aerobic cancer cells that are in closer proximity to the vasculature. The lactate produced by hypoxic cancer cells during glycolysis is exported by monocarboxylate transporter (MCT)-4 and taken up by aerobic cancer cells through MCT-1. Lactate has a multi-modal function in aerobic cancer cells, not only serving as an energy source when converted to pyruvate by LDH-B, but also acting as a buffer against oxidative stress when NADH is generated in the conversion process (Martinez-Outschoorn et al., 2014b).

Immuno-evasion refers to the ability of cancer cells to avoid immune detection and resist destruction by immune cells. This has been attributed to abnormal changes in, or complete loss of certain surface antigens on cancer cell membranes. Loss of function mutations in the gene encoding the Fas receptor of cancer cells have been reported, rendering these Fas-refractory cancer cells resistant to destruction by CD8⁺ effector T-cells. The upregulated production of decoy receptors for the Fas ligand has also been observed (Fouque et al., 2014). Equally culpable is the tumor microenvironment wherein glycolytic energy generation has been shown to cause localized acidification and consequent suppression of effector T-cell activity (Calcinotto et al., 2012). Alternatively or concurrently, regulatory T-cells that are inherently immunosuppressive are recruited to the tumor site (Mougiakakos et al., 2010). Cancer cells also secrete TGF- β . This further encourages lymphocyte differentiation into regulatory T-cells while simultaneously blocking NK activation

receptor expression via SMAD3-dependent pathways (Gigante et al., 2012). Finally, the tumor immune landscape is exacerbated by TAMs, which are responsible for tissue remodeling, sustained pro-inflammatory and angiogenic signaling and immunosuppressive effects (Cook and Hagemann, 2013). Clearly, immune escape by cancer cells is highly complex and involves the modulation of both innate and adaptive immune responses.

The many aspects of cancer may make the disease seem insurmountable and place its cure out of reach. However, viewed optimistically, our increasing understanding of each hallmark uncovers new opportunities to counter cancer progression in a multi-modal fashion. While cancer cells subvert and contort many biological processes to survive and propagate, not every hallmark of cancer needs to be simultaneously inhibited to develop effective anticancer treatments. The blockade of two or three cancer cell characteristics has been demonstrated to suffice. Equally importantly, therapeutic interventions that target the tumor microenvironment will supplement conventional anticancer chemotherapy to undermine the support that cancer cells receive *in vivo*.

1.2. Field cancerization

Slaughter and colleagues, in a landmark study of oral squamous cell carcinoma (SCC), noticed that hyperplastic lesions frequently had a much greater lateral spread than infiltration depth. Moreover, they observed that macroscopically normal tissue that was surgically excised from the area bordering tumors already displayed histological signs of malignant transformation, as characterized by hyperplasia, hyperkeratinization and overt fibrosis. The multifocality of primary tumors in close proximity was also noted in many patients. Slaughter and colleagues thus coined the

term "field cancerization" which embodies the concept that an as-of-yet unidentified carcinogenic agent results in the establishment of a contiguous premalignant field within which multiple independent neoplasias develop and eventually progress to cancer. Field cancerization is also thought to be responsible for the frequent recurrence of tumor growth at the exact site of tumor-eradicating surgery or at the margins of the surgical sutures, indicating that a regional carcinogenic signal remains in effect even after the primary tumor has been removed (Slaughter et al., 1953). The premalignant cancerized field in oral SCC can extend almost 7 centimeters from the primary tumor (Tabor et al., 2002). The concept of field cancerization has since been observed in a variety of other cancers, including those of the skin, lung, prostate, stomach and colon (Torezan and Festa-Neto, 2013).

The molecular characterization of cancerized fields has revealed that loss of heterozygosity, chromosomal aberrations and mutation are already prevalent in tumor adjacent tissue and thus represent early events in pre-malignancy. For example, fluorescence *in situ* hybridization experiments found the tumor suppressor gene TP53 to be mutated in more than 50% of patients whose head and neck tumor margins were considered histologically normal and the presence of the TP53 mutation to be positively and significantly correlated with tumor recurrence (Brennan et al., 1995). Similarly, TP53 and K-RAS mutations were detected in samples of nondysplastic crypts from patients with colitis, suggesting that a field change had occurred that tended towards carcinogenesis (Leedham et al., 2009). In skin tissue, actinic keratoses (AK) arising from chronic ultraviolet exposure were also shown to express mutant TP53 and up to 20% of AK cases progress to malignant cutaneous SCC (Glogau, 2000; Quaedvlieg et al., 2006). Given the stochastic nature of somatic mutations, it seems highly unlikely that the entire cancerized field independently acquired the same

suite of genetic defects. It was therefore suggested that field cancerization has a clonal origin in which a single mutant cell gave rise to a contiguous pre-malignant patch (Torezan and Festa-Neto, 2013).

Epigenetics has also been implicated in the establishment of a cancerized field. DNA hypermethylation at tumor-suppressor gene promoters is a known mechanism by which apparently normal tissue are primed for oncogenesis (Asada et al., 2013; Luo et al., 2014; Ramachandran and Singal, 2012), and similar findings have been reported for the promoters of tumor-suppressive miRNAs (Ando et al., 2009). Coincidentally, the enzyme DNA methyltransferase 1 (DNMT1) was overexpressed in noncancerous tissue from patients with SCC compared to normal tissue from healthy patients, suggesting that the upregulation of DNMT1 may be responsible for promoter silencing (Arai et al., 2006). Furthermore, the increased production of reactive oxygen species (ROS) accompanying chronic low-grade inflammation has been shown to relocate DNMT1 binding from GC-poor regions of DNA to GC-rich regions such as gene promoter sequences, resulting in abnormal, cancer-associated gene silencing (O'Hagan et al., 2011). Finally, epithelial histone H3 lysine 27 trimethylation has been shown to occur within 2 weeks of DSS-induced colon inflammation in mice and precedes DNA methylation, indicating that histone methylation acts in concert with DNA methylation to suppress key regulatory genes, thereby predisposing normal tissue to oncogenic transformation (Takeshima et al., 2012).

The studies discussed so far have focused on the genetic and epigenetic features of field cancerization that are initiated in the epithelia of macroscopically normal albeit histologically atypical tissue. More recent work has demonstrated that the stroma can also be a primary determinant of field cancerization (Dotto, 2014). In cutaneous SCC, prolonged exposure to the more deeply penetrating long wave

ultraviolet radiation (UVA) can result in atrophy and aberrant ECM deposition within the dermis of the skin. These changes occur before any keratinocyte abnormalities can be detected, indicating that the field defect first occurs in the stroma, changing the cues supplied to the overlying epidermis, thus resulting in the development of skin tumors (Yaar and Gilchrest, 2007). In a transgenic mouse model of stromal-directed skin carcinogenesis, the specific deletion of CSL in dermal fibroblasts resulted in multifocal hyperproliferative skin lesions that progressed to SCC. Importantly, CSL-deficient fibroblasts admixed with primary human keratinocytes and adoptively transferred into immunocompromised mice developed loose epithelial patches with increased proliferation. This suggested the stromal-directed early transformation of previously normal epithelia (Hu et al., 2012).

Field cancerization has important implications for prophylactic therapies that arrest high-risk cellular atypia before they progress to carcinomas. The success of field-directed treatments is perhaps most evident in the prevention of AK from escalating to SCC. AK is characterized by hyperkeratosis and cellular abnormalities such as enlarged or irregularly-shaped nuclei in the basal layer keratinocytes. Although most AKs regress after 5 years, the annual risk of progression to invasive disease is between 0.15 and 80% for patients who develop multiple AK lesions. For such high-risk individuals, 5-fluorouracil, diclofenac, imiquimod, ingenol mebutate and photodynamic therapy, used alone or in combination, are applied not only to multifocal AK lesions, but also to a large swathe of the surrounding skin. Complete field clearance is achieved with these therapies in ranges upwards of 50% (Dodds et al., 2014).

Although field cancerization is not considered a hallmark of cancer, the scientific literature related to tumor fields demonstrates that it is prevalent in many

different types of cancer. Interestingly, the study of field cancerization is linked to an emerging school of thought among cancer biologists that challenges the long-standing paradigm of the somatic mutation theory (SMT). SMT proposes that cancers arise from deleterious mutations in somatic cells that accumulate over time. However, more recently, the tissue organization field theory (TOFT) suggests that cancers are primarily caused by the disruption of cellular microenvironments resulting in the development of abnormal morphogenetic fields within tissues, promoting oncogenesis. The key tenets of TOFT are the following: (1) oncogenes cannot drive tumor formation without a permissive microenvironment, and (2) the microenvironment can initiate tumor formation in the absence of carcinogenic substances or any form of oncogenic activation. Several new lines of evidence support TOFT. For example, in *Xenopus laevis*, the disruption of a glycine receptor chloride channel in a specific subset of neural crest cells resulted in melanocyte hyperproliferation and enabled their inappropriate migration and colonization of other tissues. This is reminiscent of tumor growth and metastasis, except that in this model, the cancer-like phenotypes manifested in the absence of a mutagenic trigger (Blackiston et al., 2011). Separate studies in regenerating salamander limbs implanted with tumor cells also demonstrated that embryo-like microenvironmental stimuli could revert tumor cells to normalcy, indicating that mutation and oncogene addiction could be overridden by exposure to proper morphogenetic fields (Fior, 2014; Seilern-Aspang and Kratochwil, 1962).

The enduring principle of TOFT is that different cell types within tissues and organs must be in constant intercellular communication to maintain homeostasis and normal architecture. When chemical, physical, hormonal or oxidative imbalances occur, intercellular crosstalk is compromised, and if left uncorrected over extended periods of time, tissue organization becomes disordered, eventually leading to the

appearance of rogue cells and cancer (Smythies, 2015; Tarin, 2011). TOFT appears to be a unifying explanation for the parallel observations of cancer and impaired wound healing. Cancers have been described as wounds that do not heal, and slow-healing wounds are plagued by a protracted inflammation phase, signifying a prolonged departure from tissue homeostasis (Dvorak, 2015). Finally, a corollary of TOFT is that the elimination of neoplastic lesions and secondary metastases will not eradicate cancer. Identification of the underlying field defect is critical, and unless the field defect is managed or rectified, tumor relapse is likely to occur.

The evidence presented thus far highlights the importance of field cancerization and TOFT in the design of and the approach towards cancer therapy. At present, our knowledge of field cancerization remains limited, and many cancer studies still do not consider field or microenvironmental effects.

Figure 2. Schematic diagrams of field cancerization, SMT and TOFT. Field cancerization is depicted as a radial spread of oncogenic signals arising from cancer cells that affects nearby normal epithelia and mesenchymal fibroblasts (top). A side-by-side comparison of the somatic mutation theory (SMT) and the tissue organization field theory (TOFT). SMT attributes cancer to a somatic mutation that gives a cell a proliferative advantage leading to its clonal expansion within normal tissue until a tumor eventually forms. TOFT proposes that normal tissue homeostasis is non-permissive to the emergence of cancer cells. A defect in tissue architecture and function results in the development and progression of cancer.

1.3. Cancer-associated fibroblasts: key drivers of cancer progression

1.3.1. The origins of CAFs

Cancer cells do not function in isolation *in vivo*, instead depending upon a supportive microenvironment to thrive. Indeed, the immediate surroundings of an epithelial neoplastic tumor constitute a complex milieu of cells from diverse lineages, embedded within an anomalous ECM. This is collectively termed the tumor stroma. A growing body of evidence strongly suggests that cancer progression is curbed when the tumor stroma's contribution is antagonized, and conversely, the extensiveness of tumor stroma participation is correlated with poor prognosis and survival (Tlsty and Hein, 2001). Several emerging hallmarks and enabling characteristics of cancer such as immunoevasion and inflammation, have also been attributed to the various cell types making up the tumor stroma (Hanahan and Weinberg, 2011).

CAFs are the dominant cell population within the tumor stroma. While peritumoral resident fibroblasts have been earmarked as the default cells that give rise to CAFs, several other cell types within the tumor stroma have also been known to take on a CAF-like phenotype. The debate on the origins of CAFs is thus on-going and is likely to become increasingly complex as more examples of differentiation and “trans-differentiation” into CAFs are discovered. Bone marrow-derived mesenchymal stem cells can contribute to the CAF population within the tumor stroma, with osteopontin as the transformative agent (Ishii et al., 2003). Local mesenchymal cells such as pericytes can reroute their differentiation programme to become CAFs instead. The epithelial- and endothelial-mesenchymal transitions (EMT and EndMT) of epithelial and endothelial cells respectively are also recognized as potential CAF sources. Albeit a less common scenario, smooth muscle cells and adipocytes are also able to undergo differentiation into CAFs (Anderberg and Pietras, 2009; Kalluri and Zeisberg, 2006).

Clearly, the recruitment and transformation of so many different cell types into CAFs (or at least CAF-like cells) indicate remarkable plasticity in the differentiation program of cells and perhaps undermines an absolute definition of CAFs. There is even evidence that cells within the tumor microenvironment become “educated” by the prevailing biochemical and biophysical context (Erez et al., 2010; Quail and Joyce, 2013). Hence, regardless of whether CAFs were derived from epithelia, endothelia or bone marrow cells, their final identity within tumors is a myofibroblastic cell that promotes tumor malignancy. Borrowing an analogy from ecology, the existing selection pressures of the tumor microenvironment result in the "convergent evolution" of cells to become CAFs. It has therefore been proposed that the term CAF should refer to a cell state rather than a cell type, although this remains a controversial definition.

1.3.2. CAF markers and major signaling axes

CAFs were first identified by pathologists upon the histological inspection of tumor biopsy sections, during which CAFs were found embedded within collagenous fibrotic tissue in the vicinity of the tumor epithelia. This inherent capability of CAFs to produce ECM means that they can influence the type of cancer cell-ECM interactions occurring within the tumor. Morphologically, when cultured *in vitro*, CAFs appear larger and possess more stress fibers than normal fibroblasts. More precise molecular characterization of these CAFs has confirmed that they are indeed distinct from normal fibroblasts, constitutively expressing markers such as α -smooth muscle actin (α -SMA), fibroblast activation protein (FAP), fibroblast-specific protein-1, neuronal glial antigen-2, PDGF- β receptor, vimentin, desmin, collagen 11 α 1 and microfibrillar associated protein 5 (MFAP5) while at the same time displaying genetic and

epigenetic anomalies (Anderberg et al., 2009; Bhowmick et al., 2004b; Gonda et al., 2010; Vazquez-Villa et al., 2015; Yeung et al., 2014).

Two major signaling molecules, TGF- β and PDGF are responsible for inducing cellular differentiation into CAFs and enforcing the activated secretory state of CAFs, respectively. TGF- β plays complex roles in cancer and has three known isoforms, all three of which are secreted by both the cancer cells and CAFs. TGF β signal transduction occurs via canonical Smad3 and non-canonical TAK1-mediated pathways. Presumably, cancer cells initiate TGF- β secretion into the extracellular space where the molecule acts in a paracrine, heterotypic manner on nearby normal fibroblasts (Calon et al., 2014). Normal fibroblasts do not respond to TGF- β signaling with increased proliferation, but rather undergo activation and differentiation into CAFs as evidenced by an increase in α -SMA expression. CAFs in turn become a major source of TGF- β within the tumor stroma, thus perpetuating de novo CAF transformation and accrual at the primary tumor site. Intriguingly, it has been observed that the expression of the cognate receptor for TGF- β , TGF β RII, is lost in the majority of CAFs from prostate and breast cancer resulting in increased tumor metastasis and poorer prognosis (Banerjee et al., 2014; Busch et al., 2015; Li et al., 2012b). These observations suggest that autocrine TGF- β signaling becomes redundant in mature CAFs and that the sustained expression of TGF β RII within the stroma may actually play important anti-tumor roles. Indeed, mice with conditional knockout of TGF β RII via Cre recombinase expression driven by the FSP-1 promoter (TGF β RII^{fspKO}) spontaneously developed tumors in the forestomach by week 5 post-partum. This was caused by an exaggerated inflammation response leading to DNA damage and the silencing of tumor suppressors p21, p15 and p16 expression in epithelial cells (Achmut et al., 2013). In the same TGF β RII^{fspKO} mice, mammary ductal epithelia

hyperproliferation was seen in 6-week old female mice (Cheng et al., 2005) whereas prostate neoplasias were reported in male mice at 7 weeks of age (Bhowmick et al., 2004a). These collective findings support a consensus that TGF- β signaling in fibroblasts is highly context dependent and strongly impacts stromal-dependent field cancerization. Depending on the interactions between signaling axes of normal epithelia, normal fibroblasts, cancer cells, and CAFs, TGF- β can exert both tumor-promoting and tumor-suppressive effects (Calon et al., 2014; Evans et al., 2003). Clearly, tumor stroma TGF β -signaling merits further investigation at varying stages of cancer initiation and progression.

On the other hand, PDGF secretion by cancer cells has autocrine effects but is now also known to function in a paracrine, heterotypic manner that sustains the production of ECM and growth factors by existing CAFs. Tumor cell-derived PDGF binding to CAF PDGFRs is known to induce osteopontin, hyaluronan, onco-fetal fibronectin isoform and MMP upregulation by CAFs which exert anti-apoptotic, proliferative, pro-angiogenic and pro-metastatic effects in the tumor microenvironment (Gialeli et al., 2014; Jain et al., 2007; Willenberg et al., 2012). Together, TGF- β and PDGF have far-reaching, strongly transformative effects on the tumor stroma.

1.3.3. The pro-tumor functions of CAFs

1.3.3.1. CAFs stimulate cancer cell proliferation

Independently of CAF origin or marker expression, almost all reports to date have underscored the importance of CAFs in facilitating the progression of various cancers towards malignancy. CAFs have been shown to promote hepatocellular carcinoma (HCC) cell proliferation *in vitro* and *in vivo* by a HGF-mediated paracrine signaling axis, and patients with larger HCC tumors contained more intratumoral a-

SMA-positive CAFs (Jia et al., 2013). In mice studies of prostate cancer, initiated prostatic epithelia (SV40 large T-antigen immortalised but non-tumor-forming prostatic epithelia) co-injected with primary prostatic CAFs resulted in large tumors, whereas tumors could not form with co-injection of normal fibroblasts. Interestingly, non-initiated prostatic epithelia failed to form tumors despite co-injection with CAFs, suggesting that genetic instability or anomaly in epithelial cells may be necessary for CAF-mediated tumor initiation (Olumi et al., 1999). Also, CAFs explanted from endometrial cancers could potentiate the proliferation of ECC-1 and HEC-1A endometrial cancer cell lines via stimulation of both the PI3K/Akt and MAPK/Erk growth pathways. Upregulated secretion of IL-6, IL-8, MCP-1, CCL5 and VEGF by these CAFs was coordinately detected (Subramaniam et al., 2013), indicating that CAFs signal to endothelial cells and recruit TAMs to establish an angiogenic and immune-suppressive tumor microenvironment.

1.3.3.2. CAFs supply energy intermediates

From an energetic perspective, CAFs serve as a source of metabolic fuel for cancer progression. Metabolic coupling occurs between CAFs and cancer cells wherein CAFs take on a Warburg-like preference for aerobic glycolysis and display elevated ROS production, autophagic catabolism and MCT-4 expression. In this state, CAFs generate a substantial amount of high-energy intermediates that are exported by MCT4 into the tumor microenvironment. Correspondingly, cancer cells upregulate MCT1 expression for uptake of these high energy intermediates which are then channelled towards anabolic processes such as nucleotide and protein synthesis (Martinez-Outschoorn et al., 2014b; Sotgia et al., 2011). Interestingly, cancer cells in the immediate vicinity of these metabolically-coupled CAFs have been recently found

to efficiently perform oxidative phosphorylation (OXPHOS). This contradicts the view that cancer cells bear mitochondrial defects and derive energy by glycolytic means. Hence, the inclusion of CAFs in the overall scheme of cancer metabolism accentuates the metabolic heterogeneity and flexibility of cancer cells, thus providing greater insight into the molecular mechanisms by which cancer cells sustain their energy needs for rampant growth.

1.3.3.3. CAFs remodel the extracellular matrix

Beyond mitigating stromal cell-cell interactions, CAF production of proteases allows CAFs to influence cell-ECM interactions occurring within the tumor. FAP, a widely recognized marker for the CAF phenotype, is itself a serine protease that degrades collagen (Park et al., 1999). Urokinase plasminogen activator (uPA) is another CAF-secreted serine protease that lives up to its name by cleaving plasminogen into its active form, plasmin. Once activated, plasmin digests several ECM components including fibronectin and laminin. Cancer cells in close vicinity bind plasmin with CD87 to actively degrade ECM proteins, particularly at the invading front of tumors (Santibanez, 2013). Matrix metalloproteinases (MMPs) comprise a distinct class of proteases that are also produced by CAFs and either remain membrane-bound or are secreted into the extracellular space. MMPs are so called due to their dependence on zinc for their endopeptidase activity. More than 20 MMPs have been reported to play diverse roles in cancer (Kessenbrock et al., 2010). MMPs act on a large variety of extracellular substrates. By cleaving insulin growth factor binding protein (IGFBP), MMPs liberate insulin growth factor (IGF) which strongly triggers cancer cell growth and survival. The Fas ligand, which initiates the extrinsic pathway of apoptosis, is an MMP target, demonstrating that MMPs can

confer cancer cells with protection against death signals. Moreover, ECM-sequestered VEGF, FGF2 and TGF- β are also released when MMPs degrade matrix proteins, and these can contribute to cancer angiogenesis and increase fibrosis within the tumor stroma (Egeblad and Werb, 2002). ECM remodeling by CAF-derived MMPs further alters cancer cell integrin signaling, which acts in concert with growth factor-mediated receptor tyrosine kinase signaling to block both intrinsic and extrinsic apoptosis via a Raf phosphorylation-dependent mechanism. Integrin signal transduction can also indirectly activate NF κ B pro-inflammatory pathways and suppress p53 genome curation in cancer cells, leading to the upregulated expression of chemokines and cytokines and exacerbated genomic instability, respectively (Desgrosellier and Cheresch, 2010). Finally, laminin-5 which forms the basal lamina and functions as a physical barrier to invasion and metastasis can be degraded by MMPs. However, some MMPs have inhibitory effects on the hallmarks of cancer. MMP8, for example, increased B16F10 melanoma cell adhesion to laminin-coated plates *in vitro*, while lung metastases of tail vein-injected B16F10 cells were significantly more numerous in MMP8-null mice (Gutierrez-Fernandez et al., 2008). Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMP activity (Bourboulia and Stetler-Stevenson, 2010), thus the ratio of TIMPs to specific MMP subtypes produced by cancer cells and CAFs will inevitably impact cancer progression.

1.3.3.4. CAFs elevate microenvironmental oxidative stress

Reactive oxygen species (ROS) are critical intermediates of cellular signaling and persistent elevation of ROS is detected in almost all cancers. ROS such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) are by-products of aerobic metabolism. $O_2^{\cdot-}$ and $\cdot OH$ have very short half-lives ranging

from nanoseconds to minutes, can only diffuse across short distances, and have extremely low permeability across lipid bilayers. Therefore, these ROS species are highly localized to their site of production. On the other hand, H_2O_2 has a half-life of hours to days, and can diffuse across the plasma membrane passively or via anion channels. Thus, H_2O_2 is highly suitable for a role in paracrine redox signaling. Although ROS are traditionally recognized as mutagens causing genomic instability through their interaction with the sugar backbone and bases of DNA, the nefarious effects of ROS have expanded to include the disruption of cellular signaling as a result of lipid and protein modification. (Baker and Gebicki, 1984; Tafani et al., 2016; Takahashi and Asada, 1983). CAFs manipulate microenvironmental ROS levels via their pro-inflammatory secretory signature contingent on cyclooxygenase 2 (COX-2), hypoxia-inducible factor 1 α (HIF-1 α) and nuclear factor- κ B (NF- κ B) (Erez et al., 2010; Giannoni et al., 2011). Importantly, this ROS-promoting axis in fibroblasts has been observed very early in oncogenesis (Erez et al., 2010), suggesting that the establishment of a pro-tumor niche by fibroblasts is responsible for a feed-forward descent into malignancy. The inclusion of CAF-mediated ROS signaling into the overall understanding of cancer progression has important implications for therapy. For example, initial efforts focused on the inhibition of PDGF with a single agent imatinib resulted in poor outcomes (Desjardins et al., 2007; Ostman and Heldin, 2007; Reardon et al., 2005) and clinical trials examining the effect of oral antioxidants alone on cancer were inconclusive. In contrast, very encouraging findings have been obtained following combined treatment with hydroxyurea and imatinib (Dresemann, 2005; Reardon et al., 2005). Hydroxyurea, incidentally, is a potent antioxidant (Cho et al., 2010; Koncic et al., 2011). These findings suggest that promising multimodal

strategies for cancer treatment should combine the inhibition of canonical growth factor-mediated receptor activation and redox-based signaling in CAFs.

1.3.3.5. CAFs induce therapy resistance

A deeper understanding of the hallmarks of cancer has enabled the development of many treatments, thereby allowing cancer patients to live longer after diagnosis. Currently, five pillars of cancer treatment are routinely used in hospitals around the world, namely, surgery, radiotherapy, chemotherapy, targeted therapy and, most recently, immunotherapy. Each of these treatment modules has its own merits and disadvantages and are therefore often used in combination. Even as the repertoire of anticancer therapies continues to expand, acquired resistance by tumors remains a persistent problem that severely limits the durability of each treatment.

For most solid tumors, surgery is performed to remove the primary neoplastic lesion. However, situations occur in which a cancer is considered surgically inoperable. This can happen when (1) the cancer is located within a sensitive region such as the brain; (2) the cancer's spread within an organ is extensive such that its removal would threaten a patient's life; or (3) the cancer has metastasized and formed secondary neoplasms in multiple organs. Therefore, radiotherapy, traditional chemotherapy or targeted therapy often complements surgery and is employed to shrink a tumor before surgical excision or to manage metastasis in patients with late stage cancer.

Radiotherapy involves the bombardment of the tumor and the peri-tumor region with high-energy radiation (Holley et al., 2014). Mechanistically, ionizing radiation inflicts DNA lesions directly via the formation of nucleotide base radicals or cleavage of the sugar-phosphate backbone. Indirectly, DNA damage occurs by the

overt production of ROS due to the radiolysis of water. Both direct and indirect DNA damage trigger cell cycle arrest and, eventually, apoptosis when the cellular DNA repair mechanisms fail to cope with the DNA single and double strand breaks. Importantly, upon receiving a radiation dose, DNA damage spreads to adjacent cells via connexins (e.g. connexin 43), triggering collateral cell death. Another bystander effect is the release of extracellular DNA by dead cells which functions as a chemoattractant for lymphocytes. This evokes an immune response within the vicinity of the tumor, presumably assisting in tumor clearance.

Despite the destructive on-target and bystander effects of radiotherapy, resistance to radiotherapy is frequently documented. One mechanism of radioresistance arises from the inherent architecture of solid tumors. The inner core of solid tumors is hypoxic due to tumor growth outstripping *de novo* angiogenesis. Because oxygen is required to stabilize the effects of ionizing radiation on DNA, the lack of oxygen at the tumor core confers a radioprotective effect on cancer cells located therein. Cells in the tumor microenvironment, particularly CAFs, also confer radioprotection. Prostate cancer cells cocultured with CAFs had a significantly higher colony formation capacity and survival rate than monocultured controls after radiation exposure (Josson et al., 2010). Similarly, the breast cancer cell lines MCF-7 and MDA-MB-231 acquired radioresistance when co-cultured with fibroblasts that were rendered senescent by X-ray irradiation (Tsai et al., 2009). The impact of radiotherapy on CAFs is not well understood, although TGF β , integrins and E-cadherin-mediated responses have been suggested as important players in tumor-stroma crosstalk and radioprotection (Barker et al., 2015).

Chemotherapy refers to the use of cytotoxic agents that are relatively broad-spectrum in their cell-killing specificity. Traditional chemotherapy drugs do not

selectively target cancer cells, but rather preferentially accumulate in fast-cycling, proliferative cells. Thus, the side effects of chemotherapy are generally more severe and include hair loss and gut discomfort due to the cycling status of non-cancerous cells comprising the hair follicle and gut lining, respectively. Chemotherapy drugs such as cisplatin, paclitaxel and 5-fluorouracil (5-FU) interfere with the various cellular processes engaged during the cell cycle, thus triggering cell cycle arrest and eventual cell death. Cisplatin results in the formation of dominantly intrastrand DNA adducts that activate cell cycle checkpoint proteins to halt progress beyond the G2/M phase (Kelland, 2007; Siddik, 2003). Similarly, paclitaxel stabilizes the polymerized form of microtubules, hence the affected cells' inability to break down the mitotic spindle results in activation of the spindle assembly checkpoint and cell cycle arrest (Weaver, 2014). Finally, 5-FU is a structural analogue of the pyrimidine nucleotide, uracil, which is incorporated into DNA and RNA, thereby disrupting replication and transcription (Longley et al., 2003).

Targeted therapy, on the other hand, involves the use of small molecule compounds or antibodies that bind to well-defined targets in cancer cells. Unlike chemotherapy, targeted therapy has a much higher specificity for cancer cells, hence the therapeutic indices of targeted therapy drugs are usually wider and the side effects of treatment are significantly reduced. Gefitinib and imatinib are small molecule inhibitors of the receptor tyrosine kinase activities of EGFR and PDGFR respectively, both of which are abundant in cancer cells and whose downstream signaling cascades trigger mitosis (Herbst et al., 2004; Zhang et al., 2009). Their inhibition effectively blocks tumor growth. Another subclass of targeted therapies includes hormonal therapy, a strategy that is most relevant to sex steroid hormone-sensitive tumors such as those of the breast and prostate (Risbridger et al., 2010). The estrogen analog,

tamoxifen, is an example of a hormonal therapy that is used in ER-positive breast cancer. Finally, antibody-based therapy is a form of targeted therapy that uses monoclonal antibodies to specifically neutralize a surface target on cancer cells (Scott et al., 2012). One instance of an extremely successful antibody-based targeted therapy is trastuzumab, a monoclonal antibody against the HER-2 receptor in HER-2-positive and hormone refractory breast cancer.

Unfortunately, tumors that initially respond to chemotherapy and targeted therapy eventually become refractory to first-line treatments. The mechanisms of resistance vary depending on the type of drug used, although commonly observed resistance mechanisms include the following: drug efflux, drug sequestration, compensatory signaling, pathway bypass and impaired apoptosis. Upregulation of the multidrug resistance p-glycoprotein pump, a protein that actively removes xenobiotic compounds from cancer cells, is associated with acquired resistance in a wide variety of human cancers (Fletcher et al., 2010). For drugs such as cisplatin and 5-FU that enact their cytotoxic effects by targeting DNA, the upregulation of components of the nucleotide and base excision repair mechanisms have been known to confer chemoprotection (Siddik, 2003). The sequestration of cisplatin by intracellular thiols also limits treatment efficacy, while the loss of activating enzymes can prevent prodrug conversion into its active form, as is the case with capecitabine conversion into 5-FU (Shimma et al., 2000). Mutations leading to RTK activity independent of cognate ligand stimulation can also occur, culminating in overactive PI3K/Akt and MAPK signaling in cancer cells that constantly drive proliferation. The increased expression of inhibitors of apoptosis and p53 dysfunction are other means by which cancer cells gain resistance to chemotherapy and targeted therapy (Siddik, 2003).

CAFs facilitate the acquisition of chemoresistance in a non-cell autonomous fashion. As producers of ECM within the tumor microenvironment, CAFs coordinate cancer cell:ECM interactions to buffer against chemotherapy, a phenomenon that is widely known as cell adhesion-mediated drug resistance. Collagens, laminin and fibronectin which are secreted by CAFs improve integrin engagement in cancer cells which result in their elevation of IL-6 expression via intracellular signaling cascades (Berube et al., 2005). Increased IL-6 signaling in turn promotes cancer cell survival by positively regulating the expression of the anti-apoptotic protein Bcl-XL and suppressing pro-apoptotic BIM protein expression (Paraiso and Smalley, 2013).

In addition to influencing cell:ECM interactions, CAFs also alter the biophysical state of the tumor microenvironment, rendering cancerous tissue stiffer than normal tissue (Khawar et al., 2015). Lysyl oxidase, which is produced in abundance by CAFs, crosslinks collagen fibers (Lu et al., 2012). Coupled with increased ECM secretion and MMP remodeling, the matrix architecture surrounding the tumor epithelia becomes dense and largely impenetrable to anticancer drugs (Khawar et al., 2015). Furthermore, the increase in ECM density results in elevated interstitial fluid pressure (IFP) within tumors. High intratumor IFP constricts the local microvasculature and inhibits the transvasculature leakage of drugs into tumors (Chen et al., 2016).

Soluble factor-mediated drug resistance is an alternative mechanism by which CAFs confer therapy resistance to cancer cells (Meads et al., 2009). CAF-derived TGF- β , SDF-1, HGF and IL-6 act in a paracrine manner and bind to cognate receptors on adjacent cancer cells, thus enhancing cancer cell survival responses via PI3K/Akt, MAPK and STAT3 pathway activation (Kalluri, 2016). The Snail, ZEB1, Twist and Slug group of transcription factors represents another transcriptional program that is

induced in cancer cells by CAF-derived soluble factors. These molecules are master regulators of EMT and the cancer stem cell (CSC) phenotype (Smith and Bhowmick, 2016). Post-EMT cells and CSCs are notoriously difficult to treat due to their self-renewing potential and relative quiescence which allow these cells to evade drugs that primarily target fast-cycling cells (Singh and Settleman, 2010). These observations underscore the importance of CAFs in establishing a microenvironmental niche that favours therapy resistance (Holohan et al., 2013).

Clearly, a thorough understanding of the cancer-CAF interaction has both prognostic and therapeutic value and will pave the way for better management of the disease. Having established the premise that reciprocal heterotypic cell signaling events occur within epithelial tumors, the study of either cancer cells or CAFs independently yields an incomplete picture of oncogenic symbiosis. Therefore, *in vitro* co-culture systems which more accurately model this intricate relationship should be employed.

Figure 3. Schematic diagram of tumor-CAF crosstalk. A paracrine, reciprocal signaling network exists between cancer cells and CAFs that is mediated by secreted factors including chemokines, cytokines and ROS. The two cell types mutually influence each other in a feed-forward manner that potentiates the malignant progression of cancer.

1.4. Nuclear receptors in cancer and the cancer microenvironment

NRs are among the largest known classes of transcription factors that regulate gene expression in a ligand-responsive manner. To date, 48 known members of the NR family are known in humans, of which 28 receptors have established ligand profiles; the remaining 20 receptors are classified as orphan receptors whose ligands have yet to be conclusively determined. All NRs share highly conserved domain architectures including an N-terminus activation function (AF-1) domain, a C4-type zinc finger DNA-binding domain (DBD), a hinge region, a dimerization motif and a ligand-binding domain (LBD) near the C-terminus of the protein. The DBDs of NRs recognize specific response elements within the genome; thus, gene targets of NRs may be identified by the presence of unique DNA sequences that lie in close proximity to or within gene promoter regions. The dimerization motif on NRs indicates that they mostly function as homo- or heterodimers (Class 1 and Class 2 receptors, respectively). Nonetheless, some orphan receptors may bind response elements as monomers. Unliganded Class 1 receptors are monomeric, bound to heat-shock protein (HSP) and are localized within the cytoplasm, whereas unliganded Class 2 receptors are localized within the nucleus and constitutively bind to DNA as obligate retinoid X receptor (RXR) heterodimers. The LBD is the least structurally conserved among the NRdomains; hence, these receptors are able to bind diverse and distinct sets of molecules as ligands.

The canonical mechanism by which NRs regulate gene expression is as follows: for Class 1 receptors, upon cognate ligand binding, the receptor dissociates from HSP, translocates into the cell nucleus, and binds to DNA as a homodimer. Class 2 receptors, as mentioned before, are already bound to DNA. A conformational change induced by ligand binding then allows the NR to recruit either co-activators or co-

repressors to the gene promoter region, often involving remodeling of the local chromatin structure, and the final result is activation or repression of the transcription of that particular target gene (Glass and Ogawa, 2006; Olefsky, 2001) (**Figure 4**).

Figure 4. Schematic diagram depicting nuclear receptor domain architecture and the ligand-dependent canonical regulation of gene expression. Nuclear receptors may bind DNA as monomers, homodimers or heterodimers with RXR. Upon ligand binding and association with response elements of target genes, nuclear receptors recruit co-activators or co-repressors to initiate or inhibit gene transcription.

A non-canonical mechanism of negative gene regulation by NRs has also been identified. This has been termed transrepression and is perhaps most well-studied for the glucocorticoid receptor (GR) and the family of peroxisome proliferator-activated receptors (PPARs) in pathways of the inflammatory response, although other NRs also perform transrepression (Li and Yang, 2011; Ricote and Glass, 2007). Liganded GR directly interacts with the transcription factors AP-1 and NFκB. In doing so, liganded

GR sequesters these pro-inflammatory transcription factors and represses inflammatory gene transcription independently of glucocorticoid response elements on these genes. Similar interactions with AP-1 and NF κ B have been shown for PPAR-mediated transrepression (Tan et al., 2005), although PPARs may further enact transrepression via the inhibition of JNK kinase activity (with consequent silencing of the cJun/Fos transactivation of gene expression) (Desreumaux et al., 2001) and competition for co-activators (e.g. CREB-binding protein) (Kamei et al., 1996). Post-translational SUMOylation of liganded PPAR γ has also been shown to tether PPAR γ to NCoR/HDAC3/TBL/TAB2 co-repressor complexes on gene promoters lacking PPAR response elements. These repressive structures become refractory to ubiquitination and proteosomal degradation, and gene transcription is inhibited.

The importance of NRs in cancer has largely been evaluated within the tumor epithelium itself, and much of the early work was done on steroid-binding NRs (Class 1 NRs). The estrogen and progesterone receptor (ER and PR respectively) status of breast tumors were intensely scrutinized, and, together with the human epidermal receptor 2 (HER-2), have become clinical standards for the diagnosis and treatment of breast cancer patients. Four molecular subtypes of breast cancer have been identified based on three biomarkers, namely: (triple-positive), ER+/PR+/HER-2- (luminal A), ER+/PR+/HER-2- (luminal B) and ER+/PR+/HER-2+ (triple-negative) breast tumors (Onitilo et al., 2009). Importantly, ER and PR positivity of the breast tumors indicated that cancer cell proliferation was mainly dependent on estrogen and progesterone, hence receptor activation could be blocked by administration of the respective synthetic steroid hormone analogs (e.g. tamoxifen) (Ciruelos Gil, 2014; Patani et al., 2013). Therefore, in breast cancer, steroid-binding NR status accurately predicts disease aggressiveness and stratifies patient response to chemotherapy. A similar

observation was made in male patients with prostate cancer, wherein androgen receptor signaling positively correlates with cancer cell proliferation, thus warranting the use of androgen deprivation therapy to stymie disease progression (Helsen et al., 2014; Hendriksen et al., 2006). Non-steroid-binding NRs (Class 2 NRs) have also been implicated in cancer. For example, in cutaneous carcinogenesis, PPAR α expression was down-regulated in the tumor epithelia of SCCs, whereas the expression of PPAR β was increased. In melanoma, PPAR γ activation initiated cell cycle arrest (Peters et al., 2012). This is interesting because the function of PPARs was first elucidated in metabolism, particularly, lipid metabolism. PPAR α has well-established roles in fatty acid oxidation, PPAR β is paradoxically involved in both adipogenesis and fatty acid oxidation, and PPAR γ has definitive importance in maintaining the adipocyte terminal differentiation program (Bastie et al., 1999; Desvergne et al., 2006; Desvergne and Wahli, 1999; Rosen et al., 2000). Crosstalk between the PPARs and the vitamin D receptor (VDR) has also been proposed in cancer, and the inhibition of cancer cell proliferation, invasion, metastasis and drug resistance is observed upon the pharmacological activation of specific PPARs and VDR, independently or in combination (Matsuda and Kitagishi, 2013). The retinoic acid receptor, whose ligand and activity are potent stimuli for cellular differentiation during organismal development, has also demonstrated anticancer activity by acting directly on cancer cells (Das et al., 2014). Taken together, these findings demonstrate that the function of a particular NR in normal physiology is equally important in disease. NRs thus present a novel front in the search for salient therapeutic targets in various cancers.

Much less is known about the NR status of the tumor microenvironment. This is in no small part due to the dynamism and heterogeneity of the various constituents of the tumor stroma. However, without a comprehensive understanding of the

activities and functions of NRs within the tumor stroma, it becomes difficult to access NR-directed anticancer interventions that deprive cancer cells of the supportive structure that is provided by the tumor stroma.

The dominant cell type within the tumor stroma, as mentioned earlier, is the CAF, which is best characterized by its marker expression and pro-tumorigenic secretory profile. For CAFs to sustain this pro-tumorigenic phenotype, a fundamental biological program must be in place. Indeed, transcriptional regulation is altered in CAFs. A transcription factor array performed on breast cancer CAFs and fibroblasts exposed to conditioned medium (CM) of the breast cancer cell line MDA-MB-231 identified RAR and VDR activity to be significantly increased, alongside perturbations to the activities of other non-NR transcription factors (Siletz et al., 2013). This is one of the earliest instances of the definition of breast cancer CAFs based on their prevailing transcriptional network. Another independent study detected a complete loss of expression of three orphan NRs (DAX-1, $ERR\beta$ and $ROR\beta$) in breast tumor CAFs obtained from nine patients compared to the NR expression in fibroblasts from normal adjacent breast tissue. Conversely, the NR, LRH-1, was detected exclusively in CAFs. Contrary to the earlier report, VDR expression was low in these breast cancer CAFs. $TR\beta$, $PPAR\gamma$ and $ROR\alpha$ expression was also reduced in CAFs (Knower et al., 2013). Both studies on breast cancer CAFs emphasized a strong link between the NR profile in CAFs and enhancement of breast cancer cell malignancy. In addition to breast tumors, the importance of NR expression in CAFs has also shown in prostate cancer. The PR positivity of prostate stromal cells is associated with a reduction of prostate cancer cell migration and invasion. This was proposed to occur via the PR-mediated suppression of IL-6 and SDF-1 secretion by the prostate stroma (Yu et al., 2014). The same authors also reported earlier that PR inhibits prostate stromal cell

proliferation (Yu et al., 2013b). Hence, the PR status of prostate stromal cells enacts a two-pronged blockade to prostate cancer carcinogenesis: first, by limiting the expansion of tumor-supportive stromal elements, and second, by limiting prostate cancer cell invasion. These studies, albeit limited, underscore the pertinence of profiling CAFs based on their NR expression. Evidently, the NR status of CAFs impacts not only CAF behaviour but also the behavior of the tumor epithelia, likely by modifying of the tumor-CAF paracrine signaling network.

Table 1. Nuclear receptor standardized nomenclature and their ligands

Gene	Nomenclature	Abbreviation	Ligands
Thyroid hormone receptor	NR1A1*	THR α	Thyroid hormones
	NR1A2*	THR β	
Retinoic acid receptor	NR1B1*	RAR α	Retinoic acid
	NR1B2*	RAR β	
	NR1B3*	RAR γ	
Peroxisome proliferator-activated receptor	NR1C1*	PPAR α	Fatty acids, leukotriene B ₄ , fibrates
	NR1C2*	PPAR β/δ	Fatty acids
	NR1C3*	PPAR γ	Fatty acids, prostaglandin J ₂ , thiazolidinediones
Reverse erbA	NR1D1	Rev-erb α	Orphan
	NR1D2	Rev-erb β	Orphan
RAR-related orphan receptor	NR1F1*	ROR α	Cholesterol, cholesteryl sulphate
	NR1F2	ROR β	Retinoic acid
	NR1F3	ROR γ	Orphan
Liver X receptor	NR1H2*	LXR β	Oxysterols, T0901317, GW3965
	NR1H3*	LXR α	
Farnesoid X receptor	NR1H4*	FXR	Bile acids, fexaramine
Vitamin D receptor	NR1I1*	VDR	Vitamin D, 1,25-dihydroxyvitamin D ₃
Pregane X receptor	NR1I2*	PXR	Xenobiotics, 16 α -cyanopregnenolone
Constitutive androstane receptor	NR1I3*	CAR	Xenobiotics, phenobarbital
Human nuclear factor 4	NR2A1	HNF α	Orphan
	NR2A2	HNF γ	Orphan
Retinoid X receptor	NR2B1	RXR α	Retinoic acid
	NR2B2	RXR β	
	NR2B3	RXR γ	
Testis receptor	NR2C1	TR2	Orphan
	NR2C2*	TR4	Orphan

Gene	Nomenclature	Abbreviation	Ligands
Tailess-related receptor	NR2E2	TLX	Orphan
Photoreceptor-specific nuclear receptor	NR2E3	PNR	Orphan
Chicken ovalbumin upstream promoter-transcription factor	NR2F1	COUP-TFI	Orphan
	NR2F2	COUP-TFII	Orphan
ErbA2-related gene-2	NR2F6	EAR2	Orphan
Estrogen receptor	NR3A1	ER α	Estradiol-17 β , tamoxifen, raloxifene
	NR3A2*	ER β	Estradiol-17 β , other synthetic compounds
Estrogen receptor-related receptor	NR3B1	ERR α	Cholesterol
	NR3B2	ERR β	Diethylstilbestrol, 4-OH tamoxifen
	NR3B3	ERR γ	Diethylstilbestrol, 4-OH tamoxifen
Glucocorticoid receptor	NR3C1*	GR	Cortisol, dexamethasone, RU486
Mineralocorticoid receptor	NR3C2	MR	Aldosterone, spiro lactone
Progesterone receptor	NR3C3*	PR	Progesterone, medroxy-progesterone acetate, RU486
Androgen receptor	NR3C4	AR	Testosterone, flutamide
NGF-induced factor B	NR4A1	NGFIB	Orphan
Nur related factor 1	NR4A2*	NURR1	Orphan
Neuron-derived orphan receptor 1	NR4A3*	NOR1	Orphan
Steroidogenic factor 1	NR5A1	SF-1	Orphan
Liver receptor homologous protein 1	NR5A2	LRH-1	Orphan
Germ cell nuclear factor	NR6A1	GCNF	Orphan
DSS-AHC critical region on the X chromosome, gene 1	NR0B1	DAX1	Orphan
Short heterodimeric partner	NR0B2	SHP	Orphan

*denotes dimer formation with RXRs (NR2B1, NR2B2, NR2B3)

1.5. Hypotheses, experimental aims and general approach

We hypothesized that transcription-based networks in CAFs are crucial for enabling their pro-tumorigenic functions (**Hypothesis 1**). Because NRs represent a large class of druggable transcription factors, the profiling of NRs from CAFs in SCC tumors would allow the identification of novel targets for the attenuation of SCC malignancy. Analogous to the oncogene and tumor suppressor gene logic, we proposed the following roles for NRs in CAFs and set out to experimentally test them: (1) NRs that are highly expressed in CAFs relative to normal fibroblasts (FIBs) are supportive of malignant progression and their genetic or pharmacological silencing might have inhibitory effects on SCC hallmarks. (2) NRs deficient in CAFs relative to FIBs have tumor repressive properties, and their re-expression or activation might inhibit malignant progression. The same logic can be applied to the evaluation of other transcription factors whose expression pattern is associated with CAFs or a CAF-like phenotype in fibroblasts. Given the strong influence of TGF β signaling in fibroblasts, we decided that it was also necessary to compare the expression of TGF β receptor II (TGF β RII) and its downstream effectors Smad3 and TAK1 in FIBs and CAFs.

To build a transcriptional profile for SCC CAFs, we will first analyze their expression of all 48 NRs (**Aim 1**). This can be achieved by the qPCR analysis of CAF mRNA from archived patient SCC tumor biopsies and/or CAFs explanted from SCC tumors. Patient samples are formalin-fixed, paraffin embedded and H&E stained, thus enabling the identification of histological features that show a visually obvious segregation into tumor epithelial and stromal compartments. Then, laser capture microdissection (LCM) will be used to recover CAFs residing within the tumor stroma. The microdissected CAFs will then be lysed to obtain mRNA and probed for their expression of NRs. Because the NR profile of SCC CAFs is currently unknown,

and especially since SCCs are not considered hormone-sensitive cancers, obtaining this NR profile will grant theranostic insight into the microenvironmental mediators of SCC malignancy.

Next, we will explore how modulation of the CAF NR status impacts the transcriptome of SCC cells in co-culture (**Aim 2**). This will entail co-culturing the malignant SCC cell line, A-5RT3, with FIBs, CAFs and NR overexpressing or knockdown CAFs (CAF-NR-OE/KD), followed by microarray analysis of mRNA from the A-5RT3 cells. Mining of the microarray data will help identify SCC hallmarks that respond to the modulation of specific NRs in CAFs. This will guide us in designing functional tests to validate the non-cell autonomous effects of CAF transcriptional modulation. At the same time, conditioned medium from the co-cultures will be assayed for their chemokine/cytokine profile and extracellular ROS levels. Together with the microarray data, these assays will link CAF NR modulation with a resultant paracrine signaling network that may explain changes in the SCC transcriptome.

In examining the literature, we noted that many drug targets are evaluated based on their effect on a single phenotypic outcome. In doing so, certain merits of the drug target may go unnoticed. For example, the targeting of a kinase may not elicit cell cycle blockade but might instead inhibit cancer cell invasiveness. A screen based on anti-proliferative effects would therefore prematurely exclude this drug target from further development. Therefore, based on the hallmarks identified under Aim 2, we will perform functional assays to obtain a multi-parametric readout of A-5RT3 malignancy when co-cultured with FIBs, CAFs and CAF-NR-OE/KD (**Aim 3**). Of particular interest would be the ability of CAF-NR-OE/KD to augment SCC resistance to chemotherapy.

Genetically manipulating the NR expression of CAFs remains less feasible in a clinical setting than the prescription of small molecules targeting NRs. Therefore, building on the findings of Aim 3, we will evaluate whether small molecule agonists or antagonists of candidate CAF NRs can replicate the tumor-suppressive effects of CAF-NR-OE/KD (**Aim 4**). This will be verified *in vitro* and using A-5RT3:CAF xenografts in immunodeficient mice. Once again, a multi-parametric phenotypic readout will be used to demonstrate treatment efficacy. Optimistically, the use of these NR-directed compounds in combination with conventional chemotherapy regimens will yield additive or synergistic anti-tumor effects. Since many NR-directed compounds have displayed impressive success in the amelioration of various pathologies including metabolic disorders and diseases associated with inflammation, our approach will allow their repurposing for cancer treatment.

Our interest in ROS also led us to formulate a second hypothesis that diffusible ROS within the tumor microenvironment are responsible for the field cancerization of adjacent normal epithelia and fibroblasts (**Hypothesis 2**). The impact of ROS on tumor epithelia, CAFs, normal epithelia and FIBs will be studied (**Aim 5**). Exogenous ROS or their removal by antioxidants in cell culture and mouse tumor xenograft experiments will grant insight into cellular responses to ROS and shed light on the oxidative generation of a premalignant field defect favoring tumorigenesis. Subsequently, we will pursue a mechanistic understanding of how ROS might function as a causative agent of field cancerization. (**Aim 6**). Given the overarching influence of TGF β in the microenvironment of tumors, the interaction between ROS and TGF β signaling as a potential explanation for field cancerization will be explored.

Figure 5. Summary of hypotheses, experimental aims and general approaches

Chapter 2: Materials and Methods

2.1. Human tumor samples

Human SCC biopsies along with their paired peri-normal tissues were provided by the National Skin Center, Singapore. CAFs and FIBs were isolated by laser capture microdissection and subjected to protein and RNA extraction for immunoblotting and real-time PCR analyses, respectively. The study was approved by the National Healthcare Group Domain-Specific Review Boards (NHG-DSRB). All tumor samples were de-identified prior to the analyses.

2.2. Animal experiments

All animal studies were approved by the Institutional Animal Care and Use Committee of Nanyang Technological University (ARF-SBS/NIE-A0216AZ). NSG mice were obtained from Jackson Laboratories maintained under a 24-hr light-dark cycle and provided with an adequate supply of normal chow and drinking water. Six-week old NSG mice (n=4 per treatment) were subcutaneously injected with the appropriate cell types resuspended in 50 μ L of growth factor-reduced Matrigel (BD, USA). Tumor growth was monitored for the required length of time or until the tumor diameter approached 20 mm.

2.3. Cell culture

Human primary keratinocytes were obtained from ATCC and maintained in Keratinocyte-SFM (Life Technologies, USA). HaCaT, II-4, A-5 and A-5RT3 were obtained from the German Cancer Research Center and maintained in DMEM supplemented with 4.0 mM L-glutamine, 11 mg/mL sodium pyruvate, 3.7 g/L sodium bicarbonate and 10% FBS. FIBs and SCC CAFs were purchased from Asterand Bioscience. Fibroblasts were maintained in FibroGRO-LS (Millipore, USA); SCC

CAFs were maintained in DMEM supplemented with 4.0 mM L-glutamine, 11 mg/mL sodium pyruvate, 3.7 g/L sodium bicarbonate and 5% FBS. Cells were maintained at 37°C, under 5% CO₂, in a humidified incubator. All primary cells were used at low passage numbers (3-5 passages) and were sub-cultured when they reached 70-80% confluence. Sub-culture of fibroblasts and CAFs was performed with 0.05% trypsin in EDTA pH 7.4. Sub-culture of A-5RT3 was performed with 0.25% trypsin in EDTA pH 7.4. All cell lines were tested to exclude mycoplasma contamination and were authenticated against the ICLAC database.

2.4. Supplemented serum-free organotypic co-culture (SF-OTC) medium

The SF-OTC medium used was brought to a final working concentration of 75% low glucose DMEM, 25% Ham's F-12 nutrient solution, 0.25% penicillin/streptomycin, 5 µg/mL insulin, 2 ng/mL EGF, 2 ng/mL TGF- α , 0.4 µg/mL hydrocortisone, 100 pM cholera toxin, 100 µM adenine, 1 mg/mL albumin, 10 µM serine, 10 µM carnitine, 10% Fetal Bovine Serum, 1 µM isoproterenol, 50 µg/mL ascorbic acid, 1 µM α -tocopherol and 0.05 mM ethanolamine.

2.5. Laser-capture microdissection (LCM)

Formalin-fixed, paraffin-embedded (FFPE) SCC biopsies from ten patients (A-J) were analyzed by the National Skin Centre, Singapore, using qPCR. All ten patients were male, aged between 25 and 55 years, and diagnosed with SCC. Of these ten patients, patients A, C, G, and J showed signs of stroma invasion and metastasis. LCM was performed using the PALM Microbeam instrument (Carl Zeiss, Germany) to obtain SCC stromal fibroblasts. In this procedure, a focused laser beam is applied to outline tumor stromal regions containing SCC stromal fibroblasts of FFPE patient SCC tissue

sections stained with hematoxylin and eosin. A pulsed laser was subsequently used to catapult the stromal fibroblasts from the tissue sections and into 0.5mL microcentrifuge tubes fitted with opaque AdhesiveCaps (Carl Zeiss, Germany) for downstream RNA isolation and subsequent NR profiling via RT-qPCR.

2.6. RNA isolation

To isolate total RNA from stromal fibroblasts of the FFPE SCC sections that were harvested by LCM, a RecoverAll total RNA isolation kit was used according to the manufacturer's protocol (Ambion, USA). Briefly, stromal fibroblasts were treated with lysis buffer containing protease and DNase and then heated at appropriately elevated temperatures (50°C for 15 min, followed by 80°C for 15 min) to liberate the total RNA. The quality of total RNA was assessed using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., USA), and only RNA samples with 260/280 and 260/230 absorbance ratios of ≥ 1.8 and ≥ 2.0 respectively were used in downstream experiments. Reverse transcription and amplification of total RNA were performed using the Full Spectrum Complete Transcriptome RNA Amplification Kit (System Bioscience, USA). Concisely, 100 ng of total RNA from stromal fibroblasts was mixed with a universal primer mix and reverse transcriptase enzyme to perform first-strand cDNA synthesis. The proprietary universal primer mix facilitates the high-fidelity maintenance of relative transcription levels for each RNA species in sample lysates. Second-strand synthesis and amplification were then completed using Taq polymerase and proprietary Full Spectrum PCR primers that allow the unbiased and consistent amplification of all transcripts. To isolate the total RNA from *in vitro* CAF and FIB cultures, the cells were lysed with Trizol Reagent (Life Technologies, USA); RNA was then obtained using chloroform extraction and ethanol precipitation, as per

the manufacturer's protocol. Quality of total RNA was assessed with the NanoDrop Spectrophotometer (NanoDrop Technologies Inc., USA) and only RNA samples with 260/280 and 260/230 absorbance ratios of ≥ 1.8 and ≥ 2.0 , respectively, were used in downstream experiments. An iScript cDNA synthesis kit (Bio-Rad, USA) was used to perform reverse transcription using 100 ng of total RNA as template.

2.7. RT-qPCR

ProbeLibrary (Roche Applied Science, Switzerland) was used for RT-qPCR profiling and the verification of NR status in stromal fibroblasts, CAFs and NR-modified CAFs. The PerfeCTa SYBR Green FastMix (Quanta Bio, USA) was used in qPCR for non-NR transcripts. Forward and reverse primer pair sequences for each NR gene were based on the list recommended by Roche Applied Science (www.universalprobelibrary.com) (Table 2). Primer sequences for other genes are listed in Table 4. Changes in gene expression relative to CAF were determined using the $\Delta\Delta C_t$ method after normalization against the housekeeping genes ribosomal protein L27 rRNA (RPL27) and TATA-box promoter (TBP). Normalization was performed using the geometric averaging of multiple internal control genes (geNORM). Heatmaps were constructed using Orange Canvas software (University of Ljubljana).

Table 2. List of primers and probes

Gene	Nomenclature	Abbreviation	Sequence (5' to 3')	Probe No.
Thyroid hormone receptor	NR1A1	THR α	F: ccgctgtcacttgtgagg R: gctgtcataattgcaggaatagg	58
	NR1A2	TR β	F: ttggcatggcaacagattt R: ccggttctcctctatcagctt	42
Retinoic acid receptor	NR1B1	RAR α	F: aggctaccactatgggtca R: gttgatgatgcagttctgtcc	89
	NR1B2	RAR β	F: ttgtgttcacctttgccaac R: cggttcctcaaggctcctg	51
	NR1B3	RAR γ	F: cgctccatccaagagactg R: gagggaactgggcccgtag	70
Peroxisome proliferator-activated receptor	NR1C1	PPAR α	F: agattcaggagaggcaagaaaa R: gcatcagcttcatgggtga	56
	NR1C2	PPAR β/δ	F: ctcaatggggaccacag R: agctccgggagaggctctg	11
	NR1C3	PPAR γ	F: gacctgaacttcaagagtacaaaa R: tgaggcttattgtagagctgagtc	39
Reverse erbA	NR1D1	Rev-erb α	F: cctggactccaacaacaaca R: tagagggaattcagggtggt	52
	NR1D2	Rev-erb β	F: agaacaagaatagttacctgtgcaac R: ggagacttactcattggacaacc	69
RAR-related orphan receptor	NR1F1	ROR α	F: tacatgtgaaggctgcaagg R: ggagtaggtggcattgcttt	2
	NR1F2	ROR β	F: gatcactcacgccatccaat R: aaccacttccaagcaacctg	66
	NR1F3	ROR γ	F: gcgctccaacatcttctccc R: caggttccacatctcc	6
Liver X receptor	NR1H2	LXR β	F: acagcaaggacgactccac R: actcgaagatggggtgatg	39
	NR1H3	LXR α	F: agtgtcggcttcgcaaat R: ggccgcatctgttctctgac	52
Farnesoid X receptor	NR1H4	FXR	F: acctgtgaggggtgtaaaggt R: gccccgttttacacttg	2
Vitamin D receptor	NR1I1	VDR	F: atcggcatgatgaaggagtt R: ttccgcttcaggatcatctc	6
Pregane X receptor	NR1I2	PXR	F: tcgccattactctgaagtctac R: catgatcttcaggacaagaacc	57
Constitutive androstane receptor	NR1I3	CAR	F: gctggcatgaggaagaca R: tgcacagggtgttgcctgtg	29
Human nuclear factor 4	NR2A1	HNF α	F: gagatccatggtgttcaagga R: gtgccgagggacaattgtagt	68
	NR2A2	HNF γ	F: tcttggtgaatgggctaaa R: agctctcaacagtgccacct	8
Retinoid X receptor	NR2B1	RXR α	F: acatgcagatggacaagacg R: gagagccccttgagtcag	26
	NR2B2	RXR β	F: agtccccccaggattctc R: caggagagtgacactgttgagtt	66
	NR2B3	RXR γ	F: tgtcatggcatgaagagg R: cctcactctcagctcgctct	82
Testis receptor	NR2C1	TR2	F: cctgatctgtctgcacaacac R: ttgtccttggctcggaagt	17
	NR2C2	TR4	F: ggccgaaatcctgaggtg R: tcggtggagattatctggatg	80
Tailless-related receptor	NR2E2	TLX	F: ggacctatgtctgcaaatctgg R: ttctgtgcgtcttctccac	75
Photoreceptor-specific nuclear receptor	NR2E3	PNR	F: ggtggcctgtggagtcag R: caatctgtccaaactcagttccatgctgctg ttcacgtcaga	25
Chicken ovalbumin upstream promoter-transcription factor	NR2F1	COUP-TFI	F: atcgtgctgttcacgtcaga R: gctcctcacgtactcctcca	2
	NR2F2	COUP-TFII	F: gccatagtcctgttcacctca R: aatctcgtcggctggttg	36
ErbA2-related gene-2 Estrogen receptor	NR2F6	EAR2	F: ccaggagcagggtggacaa R: aaccagtgcaccattgataaaa	46
	NR3A1	ER α	R: tctcttcggctctttctgatac F: cgagagttaaaactccaacacaaa	69
	NR3A2	ER β	F: cgagagttaaaactccaacacaaa R: tcgctgtgaccagagggtg	15

Gene	Nomenclature	Abbreviation	Sequence (5' to 3')	Probe No.
Estrogen receptor-related receptor	NR3B1	ERR α	F:ggcggcagaagtacaagc R:attcactggggctgctgt	3
	NR3B2	ERR β	F:agagaggcaggcagatctca R:gtgagccagagatgctttcc	79
	NR3B3	ERR γ	F:ccagccaaaaagccattg R:gacagtagggtcaggcatgg	83
Glucocorticoid receptor	NR3C1	GR	F:agttttctcaaaagagcagtg R:caatcattcctccagcaca	56
Mineralocorticoid receptor	NR3C2	MR	F:acaggtagacggcgagagac R:tcaggagactgtgtagcc	58
Progesterone receptor	NR3C3	PR	F:ggatgagctaatggtgttgg R:gattcttcacccgtgttca	58
Androgen receptor	NR3C4	AR	F:gccttgctctctagcctcaa R:gtcgtccacgtgtaagttgc	14
NGF-induced factor B	NR4A1	NGFIB	F:acagcttgctgtcgtatgc R:ggttctgcagctcctccac	34
Nur related factor 1	NR4A2	NURR1	F:gcccatgtcgactccaac R:actcattgatagtcagggttcg	64
Neuron-derived orphan receptor 1	NR4A3	NOR1	F:catcagcagccatccattc R:ctgcttgaagtacatggaggtg	19
Steroidogenic factor 1	NR5A1	SF-1	F:gcaggtgcatggtcttcaa R:agttctgcagcagcgtcat	10
Liver receptor homologous protein 1	NR5A2	LRH-1	F:tgtctgaaattcttggtgctctt R:tgaattgttctggacacctt	11
Germ cell nuclear factor	NR6A1	GCNF	F:aagtactgccctccgatg R:tggaaactgtgatagaggtagatga	58
DSS-AHC critical region on the X chromosome, gene 1	NR0B1	DAX1	F:ccatcaagtgtcttttcca R:cctgaatgtacttcagcactg	67
Short heterodimeric partner	NR0B2	SHP	F:tgtgtctggagtccttctg R:ccagtgagcctcctgctg	1
Controls				
18S ribosomal RNA		18S rRNA	F:gtaaccggtgaacccatt R:ccatccaatcgtagtagcg	48
TATA Box Promoter		TBP	F:gctggtatcgggagttgg R:actggcctgggtctctagag	30

Table 4: List of primer sequences

ChIP primers	Forward (5' to 3')	Reverse (5' to 3')
AP1(-12)	TTCCGGCTTAGGAGGAGCAC	CCGCCGCTAGCCGAGCAGC
Smad3(-171)	GGAACCTCTGAGAAAAAA	TGAGGGTCCCGCCTCATCCG
AP1(-262)	ACCGGAAGCCTAGATCCCTCTG	TCCAGGTTACCCTTCCAAGCTG
Negative control (-2016)	AGGCTGGGTACAGTGGCTCATG	GCTCGTCGGTCGCCAGGC

qPCR primers		
ANGPTL4	CTCCCGTTAGCCCCTGAGCG	AGGTGCTGCTTCTCCAGGTG
BCL-2	GAAGTGGGGGAGGATTGTGG	CCGGTTCAGGTACTCAGTCA
C/EBP β	CTTCAGCCCGTACCTGGAG	GGAGAGGAAGTCGTGGTGC
cMET	TGAAGTAATGCTAAAATGCTGG	CCTATGGCAAGGAGCAAAGA
CXCL12	CAGCCTGAGCTACAGATGCC	CTGAAGGGCACAGTTTGGAG
EGFR	AAGGAAATCCTCGATGAAGCCT	TGTCTTTGTGTCCCGGACATA
EPHA1	CAAGGACGCAGAGACACTGAC	CACCGTGGTTACCTTCTGGA
ERBB2	CTCTTAGACCATGTCCGGGAA	AGCCCGAAGTCTGTAATTTTGAC
FYN	TTCAATTCCGTAGCCAGCTGC	ACAGAGGAGGTTCCGATTTGGG
JAK2	GTATCCACCCAACCATGTCTTC	GTTGCTGCCACTGCAATACC
JAK3	AGGACCGGCCATCATTCAG	AGCAGTGAAGCATGAGTCTC
JUN-B	TACCACGACGACTCATACACA	CGCTTTGAGACTCCGGTAGG
KLF-5	CTGAGAACTGGCCTCTACAAATC	TGGTGTGAGCTGAATATACTGGT
Lgr5	ACGCTCTGACATACATTCCCA	GATTGAAGGCTTCGCAAATTCTG
MAPK14	CAGCAGATTATGCGTCTGACA	TCAGGATCGTGGTACTGAGCA
MDM2	AGCCTGGCTCTGTGTGTAATA	CCTGATCCAACCAATCACCTGA
MMP1	ACTCTGGAGTAATGTCACACCT	GTTGGTCCACCTTTTCATCTTCA
MMP3	CGGTTCCGCTGTCTCAAG	CGCCAAAAGTGCCGTGTCTTCA
MMP9	ACCTCGAACTTTGACAGCGAC	GAGGAATGATCTAAGCCCAGC
MMP13	TTTCAACGGACCCATACAGTTTG	CATGACGCGAACAATACGGTTA
Myc	CCACAGCAAACCTCCTCACAG	GCAGGATAGTCCTCCGAGTG
P15	GGTGCAGAGCTGTCGCTTTCAG	TCAGCCAGCCAGTTGGTTTCAC
P21	CCTGTCACTGTCTTGTACCCT	CCCGCAGTATCTTGCCCTCC
PPAR β/δ	AGGAGCCATTCTGTGTGTGA	TCCTGCCAGCAGAGAGTGAT
Smad3	GAACGTCAACACCAAGTGCAT	ACGCAGACCTCGTCTCTCT
SOCS	GCTTTTTCGCCCTTAGCGTG	GAGGCAGTCGAAGCTCTCG
STAT3	ATCACGCCTTCTACAGACTGC	CATCCTGGAGATTCTCTACCACT
STAT5b	TAAGAGGTCAGACCGTCGTGG	GCTGCCATGAACGATCACCA
TAK1	GTTCCGTGTAAGGGCTTTGA	GAGCAGCTGCCACTTACCTTTACA
TGF β R2	CGTGGAGTCGTTCAAGCAGACG	CCGCACCTTGGAAACCAATGG
VHL	GAGTCCGGCCCGGAAGAGTCCG	CATCGTGTGTCCCTGCATCTCT

2.8. Protein extraction, immunoblotting and immunofluorescence staining

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA). Protein extracts were resolved using 10% SDS-PAGE and transferred onto Immobilon-FL low-fluorescence membranes (Merck Millipore, USA) for immunoblotting. The primary antibodies used against mouse and human protein targets were as follows: RAR β , PPAR β/δ , VDR, GR, AR, PCNA, GLUT1, and β -tubulin (Santa Cruz, USA); TGF β RII, TAK1, Smad3, GPX1, and NF κ B (Cell Signaling, USA); cisplatin adducts (Abcam). Infrared dye-conjugated secondary antibodies (IRDye) were obtained from LI-COR, USA. Protein bands were detected using an Odyssey CLx infrared imaging system (LI-COR, USA). For immunofluorescence staining, Ki67 and E-cadherin antibodies were obtained from Dako, Denmark and Cell Signaling, USA, respectively. Dyelight fluorescent-conjugated antibodies were obtained from Thermo Scientific, USA. The terminal deoxynucleotidyltransferase-UTP nick-end labeling (TUNEL) assay (Life Technologies, USA) was used for immunofluorescence detection of apoptosis in tissue samples. All immunofluorescent samples were imaged under an LSM710 confocal microscope (Carl Zeiss, Germany).

2.9. Cell transfection

Cells were transfected 24 hours after seeding. Fibroblasts were transfected with Dharmafect SMARTpool small interfering RNAs (siRNAs) targeting selected NR transcripts, TGF β RII, Smad3, TAK1 and NF κ B using DharmaFECT1 according to manufacturer's recommendation (Dharmacon). Briefly, a transfection mixture containing 25 nM final concentration of siRNA was constituted from siRNA and DharmaFECT reagent and applied to cells in complete medium. For over-expression

of NRs in CAFs, plasmids encoding selected NRs were purchased from Missouri S&T cDNA Resource Center and Addgene (Addgene, USA) based on availability. FugeneHD (Promega, USA) was used as the vector for plasmid delivery into CAFs. Briefly, CAFs were treated with a FugeneHD:plasmid ratio of 3:1 using 0.55 µg of plasmid cDNA. NR knockdown/overexpression was verified by RT-qPCR.

2.10. Co-culture and conditioned medium experiments

Co-culture of A-5RT3 cells with FIBs, CAFs or NR-OE/KD-CAFs was carried out in 24-well Boyden Chambers containing 6.5 mm inserts with 8 µm pores. FIBs and CAFs were seeded at a density of 1.5×10^4 cells per well in their respective culture media. Boyden Chamber inserts were coated with 50 µg/mL rat-tail type I collagen in acetic acid (BD, USA), allowed to stand for 1 hour at 25°C and rinsed thoroughly with PBS to remove acetic acid. A-5RT3 cells were seeded at a density of 3×10^4 cells per insert in DMEM 10% FBS one day after CAF transfection. Cells were then co-cultured for 48 hours in SF-OTC medium, after which A-5RT3 were harvested for microarray. Conditioned medium from FIBs, CAFs and NR-OE/KD-CAFs was collected for treatment of A-5RT3. Post-seeding and transfection, culture medium was replaced with SF-OTC medium, conditioned for 48 hours and then filtered (0.2 µm, Millipore, USA) before being added to A-5RT3.

2.11. Microarray and analysis of microarray data

After 48 hours of co-culture with FIBs, CAFs or NR-KD/OE-CAFs, total RNA was isolated from A-5RT3 using Trizol Reagent (Thermo Scientific, USA) and underwent reverse transcription and amplification using Applause WT-Amp System (Nugen, UK). Post-amplification cDNA was labelled with Encore Biotin Module (Nugen, UK),

and then loaded onto GeneChip Human Gene 1.0 ST Array (Affymetrix, USA) chips for hybridisation, washing, staining and scanning. Hybridisation was performed in the GeneChip Hybridisation Oven 645 (Affymetrix, USA). Washing and staining was performed with GeneChip Fluidics Station 450 (Affymetrix, USA). Array chips were scanned with GeneChip Scanner 3000 7G (Affymetrix, USA). Raw microarray .cel files were imported into Partek Genomic Suite (v6.6) and normalised using robust multiarray averaging (RMA). ANOVA was performed for all A-5RT3:CAF co-culture samples with contrasts set against the A-5RT3:FIB sample. Genes differentially expressed by >1.5-fold between A-5RT3:CAF and A-5RT3:FIB co-culture samples were selected (302 genes) and examined in all A-5RT3: NR-OE/KD-CAFs co-culture samples (OE = over-expression, KD = knockdown). Hierarchical clustering ranked A-5RT3:NR-OE/KD-CAFs gene expression profiles from most similar to most distinct from A-5RT3:CAFWT. A list of 70 genes was defined as “inversely regulated” in five A-5RT3:NR-OE/KD-CAFs conditions (Cluster A). Gene ontology (GO)-enrichment analysis was then performed on these differentially regulated genes to identify putative biological functions altered in A-5RT3 cells by NR modification in CAF. A χ^2 test was used to assign each GO term with an enrichment score (ES). Briefly, a higher ES indicates that a larger subset of genes from a gene list is found to be associated with a particular GO term than would be expected by chance. All 302 genes were also input to the Ingenuity Pathway Analysis (IPA) software for further functional annotation and phenotypic association of these focus molecules. “Physiological system development”, “Diseases and biological functions” and “Molecular and cellular functions” were categories of interest for core analysis by the program. Enrichment of the 302 genes in each of the above three categories (and subcategories) was assessed based on p-values calculated by the analysis platform. Essentially, the p-value is tabulated using the ratio

of the number of genes within the input dataset that participate in a category of interest, to the total number of genes with known affiliations to the said category documented in the IPA knowledgebase. Statistical evaluation with Right-tailed Fisher's exact tests is then performed to test the probability that any identified affiliations may be attributed to random chance.

2.12. Invasion assays

FIBs, CAFs or NR-KD/OE-CAFs were seeded at a density of 1.5×10^4 cells per well of a 24-well plate in their respective culture media. 6.5 mm Boyden Chamber inserts with 8 μm pores were coated with 50 $\mu\text{g/mL}$ type I rat-tail collagen in acetic acid, allowed to stand for 1 hour and rinsed thoroughly with PBS to remove acetic acid. Epithelial cells at varying stages of oncogenic transformation, namely, HaCaT, HSC, II-4 and A-5RT3 cells (ranked from least to most malignant) were seeded at a density of 5×10^3 cells per insert in DMEM 10% FBS. Complete medium was replaced by supplemented SF-OTC medium at the start of the invasion assay, with CAFs conditioning the medium and providing the chemotactic gradient. Boyden Chambers were incubated at 37°C, 5% CO₂ for 16 hours, after which the Boyden Chamber inserts were removed. Epithelial cells on the top and bottom of the inserts were fixed in 1% glutaraldehyde in PBS. Epithelial cells on the top of the inserts were removed with a cotton swab. Invaded cells remaining on the bottom of the inserts were stained with the infrared dye, SYTO60 (Life Technologies, USA) in PBS (1:5000 dilution) for 5 minutes at 37°C. SYTO60 is cell permeant and fluoresces at 680 nm only upon binding to cellular DNA. Post-staining, SYTO60 fluorescence of each insert was read using the Odyssey CLx infrared imaging system (LI-COR Biosciences, USA).

2.13. Proliferation assays

Proliferating epithelial cells, alone or in co-culture with FIBs, CAFs or NR-KD/OE-CAFs for 48 hours, were analyzed by bromodeoxyuridine (BrdU) and propidium iodide (PI) staining using the BrdU Flow kit (BD Biosciences). Epithelial cells were incubated with BrdU (40 μ M) for 30 minutes, then rinsed twice with PBS to remove excess BrdU, followed by fixation in 70% ice-cold ethanol. After fixation, cells were treated with 1.5M HCl for 1 hour to denature DNA, rinsed twice with PBS to remove excess acid, and incubated with 1:100 ratio of FITC-conjugated anti-BrdU in PBS with 5 μ L PI per 1×10^6 cells. Finally, cells were subjected to FACs analysis using the Accuri C6 Flow Cytometer (BD, USA). FACs data was processed with FlowJo (Treestar, USA).

2.14. Drug treatments

In vitro: Selective agonists of PPAR β/δ (GW0742), VDR (EB1089) and GR (fluticasone propionate), and selective antagonists of AR (bicalutamide) and RAR β (LE135) were used on CAFs at 5 μ M final concentration over 48 hours of treatment (Tocris Bioscience, UK). Excess agonists/antagonists were washed off thoroughly with 3 rinses of PBS, and SF-OTC medium was replaced in the wells for conditioning by the pre-treated CAFs. The 48 hour conditioned medium of FIBs, CAFs, NR-KD/OE-CAFs or pre-treated CAFs was collected and passed through a 0.2 μ m filter to remove any cellular debris before being used to culture A-5RT3. For IC₅₀ determination, 10, 100, 1000, 10000 and 100000 ng/mL of cisplatin (Sigma Aldrich, USA) was added to A-5RT3 cultured in FIBs, CAFs, NR-KD/OE-CAFs or pre-treated CAFs conditioned media for 16 hours. *In vivo*: 6-week old NSG mice (n =4 per treatment) were subcutaneously injected with 2×10^5 A-5RT3 cells in combination with

6×10^5 CAFs resuspended in 50 μ L growth factor-reduced matrigel. Treatments were started 3 weeks after tumor xenografts were established (tumor volumes ≥ 150 mm³). For cisplatin treatment, mice received intraperitoneal or intratumor injections of 2 mg/kg cisplatin for 2 consecutive weeks. For combination treatments, intraperitoneal injection of 5 mg/kg LE135 and/or 10 mg/kg bicalutamide was administered each time cisplatin was injected.

2.15. Apoptosis assays

To determine the IC₅₀ of A-5RT3 cells subjected to the various treatments, PSVue794 at a final concentration of 10 μ M was added to cells and incubated at 37°C for 30 minutes. PSVue794 only fluoresces when it binds to phosphatidylserine residues exposed on apoptotic cells. Cells were rinsed twice with PBS before being imaged using the CLx infrared imaging system (LI-COR Biosciences, USA). The dye fluorescence intensity positively correlates with the extent of apoptosis occurring. Alternatively, apoptotic and necrotic cells were quantified using an Annexin V/PI staining kit (BioLegend, USA). Briefly, cells were incubated with FITC-conjugated Annexin V (1:100 in binding buffer) and 5 μ L of PI per 1×10^6 cells for 10 mins at 37°C followed by FACS analysis using the Accuri C6 Flow Cytometer (BD, USA). FACS data was processed with FlowJo (Treestar, USA).

2.16. Glucose uptake assays

***In vitro*:** A-5RT3 were co-cultured with FIB, CAF and NR-OE/KD-CAFs for 48 hours in SF-OTC, after which the medium was replaced with glucose-, serum- and phenol red-free DMEM containing 10 μ M of the fluorescent glucose analog, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) (Life

Technologies, USA), and incubated at 37°C, 5% CO₂ for 30 minutes. Following incubation with 2-NBDG, A-5RT3 were trypsinised, rinsed twice with PBS and analysed for glucose uptake by the Accuri C6 Flow Cytometer (BD, USA) at excitation/emission of 465/540 nm. ***In vivo***: tumor glucose uptake was measured using the 800CW-2DG IRDye (LI-COR Biosciences. Briefly, animals were fasted for 4 hours before being injected with 20nmol of 800CW-2DG IRDye and maintained under normal conditions overnight. Tumor uptake of the 800CW-2DG IRDye was measured thereafter using the Odyssey CLx infrared imaging system (LI-COR Biosciences, USA).

2.17. HPLC quantification of AMP, ADP and ATP nucleotides

To prepare for nucleotide determination, cells under indicated treatments were lysed using boiling milli-Q water to precipitate cellular proteins. Supernatant containing the nucleotides were collected for analysis. Nucleotides standards (Sigma Aldrich, USA) prepared in milli-Q water was resolved on a Shimadzu LC-20AD series Quaternary Gradient HPLC system with PDA detector using a Hypersil ODS 150 mm x 4.6 mm I.D. C18 reverse-phase column with 3 µm particle size and 5 µmHypersil ODS C18 guard column (Thermo Scientific, USA). Separation of the nucleotides were carried out with a mobile phase containing 60 mmol/L of KH₂PO₄, 0.45 mmol/L of tetrabutylammonium bromide (TBAB) and 1.26 mol/L of acetonitrile. The pH of the mobile phase was adjusted to 3.20 using concentrated phosphoric acid. Retention times of the individual nucleotides are matched to that of the respective nucleotide standards. Area under the peaks that correspond the respective nucleotides were tabulated and energy charge was calculated using the formula:

$$\text{Energy charge} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

2.18. ROS assays

Intracellular ROS: A-5RT3 were co-cultured with FIB, CAF and NR-OE/KD-CAFs for 48 hours in SF-OTC, after which the medium was replaced with serum- and phenol red-free DMEM containing 10 μ M of 5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester fluorophores (CM-H₂DCFDA) (Molecular Probes, USA) and incubated at 37°C, 5% CO₂ for 30 mins. Following incubation with CM-H₂DCFDA, A-5RT3 were trypsinised, rinsed twice with PBS and analysed for intracellular ROS by the Accuri C6 Flow Cytometer (BD, USA) at excitation/emission of 495/527 nm. The above protocol was used to assay intracellular ROS in FIBs, CAFs and NR-OE/KD-CAFs as well. *Extracellular H₂O₂:* The 48-hour conditioned medium from FIBs, CAFs and NR-OE/KD-CAFs was subjected to the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, USA) for detection of extracellular H₂O₂. In principle, 0.1 U/mL horseradish peroxidase (HRP) in Krebs-Ringer Phosphate Buffer catalyses the conversion of H₂O₂ in 50 μ L of conditioned medium into hydroxyl radicals ($\cdot$ OH), which then react with the Amplex Red reagent(10-acetyl-3,7-dihydroxyphenoxazine) to produce resorufin. Resorufin signal was detected using the Tecan SPECTRA Fluor Plus plate reader (Tecan Systems, USA) at excitation/emission of 485/590 nm.

2.19. Multiple analyte detection

48-hour conditioned medium from A-5RT3 co-cultured with FIBs, CAFs or NR-KD/OE-CAFs was filtered (0.2 μ m, Millipore, USA) and stored at -80°C until

analysis. MILLIPLEX MAP Human MMP, Human Angiogenesis/Growth Factor and Human Circulating Cancer Biomarker magnetic bead kits were used for multiplexed immunoassay detection of a selected panel of conditioned medium analytes. In each well of a 96-well plate, 25 μ L of conditioned medium was incubated with 25 μ L of assay buffer and 25 μ L of antibody-conjugated bead mixture for durations indicated in the respective kit protocols. Post-incubation, beads were washed twice with wash buffer. Biotinylated detection antibodies were added, followed by 2 hour incubation with agitation at 25°C. Subsequently, 25 μ L of streptavidin-phycoerythrin was pipetted into each well, incubated for 30 minutes with agitation at 25°C before washing twice with wash buffer. Finally, 100 μ L of sheath fluid was added to each well and plate-read was performed on the MAGPIX system (Luminex Corporation, USA). Data analysis was performed with the onboard Luminex xPonent software (Luminex Corporation, USA).

2.20. Organotypic coculture (OTC)

Briefly, 4 mg/mL of Type 1 collagen in 0.2N acetic acid (Corning, USA) was neutralized using 1N NaOH with gentle stirring on ice. Primary human dermal fibroblasts in 1x Hank's buffered saline were then added dropwise to the neutralized collagen to achieve a final density of 1.5×10^5 fibroblasts/mL of collagen. The fibroblasts in collagen were dispensed into 24 mm diameter transwell inserts and allowed to gel before being transferred into deep wells containing SF-OTC medium. Subsequently, 1×10^6 epithelial cells in SF-OTC medium were seeded onto the collagen layer and allowed to grow and stratify over three weeks.

2.21. *In vitro* tumor colony promotion protocol

Keratinocytes were seeded at a density of 1×10^5 per 35-mm dish containing complete OTC medium (Denova Sciences) at day 0. On day 1, the cultures were treated for 1 hour with the initiating agent, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or solvent control (DMSO). The cells were washed, and the medium was replaced with complete medium 24 hours prior to exposing the cells to various concentrations of H_2O_2 . After one week, cells were harvested from individual dishes and passaged at a density of 1×10^5 per 35-mm dish in H_2O_2 -containing medium. Concurrently, 2×10^4 harvested cells were embedded in 0.35% noble agar (Sigma Aldrich) and layered onto 0.7% noble agar-coated 35-mm dishes during each passage of the cells. These soft agar cultures were consistently replenished with fresh H_2O_2 -containing medium every other day. After 1 month, viable transformed colonies were stained with 1 mg/ml thiazolyl blue tetrazolium in PBS and counted.

2.22. PEG-switch assay and OxyBlot protein oxidation detection

PEG-switch assay was performed as previously described.(Burgoyne et al., 2013) Cells treated with 100nM H_2O_2 or 10 ng/mL MNNG for 24 h were lysed using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, USA). Oxidized thiols were reduced using DTT and alkylated using 2 mM PEG-maleimide with 0.5% SDS for 2 h at room temperature. The alkylation reaction was quenched using 100 mM Tris-HCl buffer pH 6.8, 4% SDS, 20% glycerol, and 0.01% bromophenol blue and 100 mM maleimide and the samples were resolved by SDS-PAGE and subjected to immunoblot. To analyze oxidatively modified PTEN and Src , the cells that had been treated with 100 nM H_2O_2 for 30 min were washed with oxygen-free ice-cold PBS containing 1 mM *N*-ethylmaleimide (NEM) and then lysed

in lysis buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 30 mM NEM). The lysates were further clarified using a QIAshredder (Qiagen). Equal amounts of proteins were mixed with NEM loading dye (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 20 mM NEM, and 0.02% bromophenol blue) and resolved by non-reducing SDS-PAGE. As a control, the proteins were completely reduced by heating in SDS-loading dye containing DTT. The separated proteins were subjected to immunoblot analysis using antibodies against PTEN and Src.

2.23. H₂O₂ and CAF-conditioned medium treatment of FIBs in vitro

FIBs and CAFs were seeded at a density of 1×10^4 cells per 35-mm dish in FibroGRO-LS (Millipore). The 48 hour conditioned medium was collected from CAFs, sterile filtered and used immediately. CAF conditioned medium (CCM), with or without 100 μ M N-acetylcysteine (Sigma) pretreatment, was added to wells containing FIBs and was refreshed daily over 3 days, after which FIBs were harvested for FACS analysis. Separately, FIBs were treated with 100 nM H₂O₂ in serum-free FibroGRO-LS for 30 min twice daily at 8 hour intervals between treatments for a maximum of 3 days. FIBs were harvested for FACS analysis 24 h after 1 day, 2 days and 3 days of H₂O₂ treatment.

2.24. In vivo tumor growth and adoptive transfer of transformed FIBs

A total of 1×10^5 HaCaT or A-5RT3 cells were admixed with 2×10^5 CAFs and 1×10^5 VybrantDiO (Life Technologies)-labeled FIBs in growth factor-reduced Matrigel (Corning), with or without 500U/mL catalase (Sigma), and implanted subcutaneously into NSG mice (Jackson Laboratory). Three weeks post-implantation, the resulting tumors were excised and dissociated using the MACS tumor dissociation kit and a

gentleMACS Octo Dissociator (MiltenyiBiotec). The tumors were then doubly stained with CellROX Deep Red (Thermo) and PE-conjugated anti-FAP antibody (Novus Biologicals) prior to FACS analysis. A total of 3×10^5 ROS^{high}/FAP^{high} FIBs were isolated and adoptively transferred into new tumor xenografts containing either HaCaT or A-5RT3 cells. Tumor growth was monitored for 3 weeks.

2.25. Chromatin immunoprecipitation (ChIP) and re-ChIP

ChIP was performed using phospho-cJUN and phospho-Smad3 antibodies (Cell Signaling), and re-ChIP was performed using a p300 antibody (Upstate Biotechnology). Briefly, FIBs were fixed in 1% paraformaldehyde and lysed for chromatin. The genomic DNA was sonicated into 200-500 base pair fragments using a handheld sonicator (Hielscher, Germany) at six 15 second pulses followed by 45 Chromatin was pre-cleared for 2 hours at 4°C using protein A-agarose beads/salmon sperm DNA (Millipore, USA), centrifuged at 3000 rpm for 5 minutes at 4°C to remove the beads, and incubated with the relevant antibodies overnight at 4°C with rotation. Subsequently, protein A-agarose beads/salmon sperm DNA was added to the chromatin samples, incubated for 2 hours at 4°C, then centrifuged at 3000 rpm for 5 minutes at 4°C. The supernatant was removed, and the beads were washed four times with rotation for 10 minutes each using high-salt buffer (50 mM HEPES pH7.9, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate). Immunoprecipitated chromatin was eluted in 50 mM Tris-HCl pH 8.0, 10 mM EDTA containing 1% SDS. Reverse crosslinking of immunoprecipitated chromatin samples was performed at 65°C overnight, followed by purification of DNA using a PCR purification kit (Qiagen, USA) and analysis by PCR. The primer sequences used are listed in Table 4.

2.26. Mitogenic factor array

The expression profiles of the secreted factors present in the 48 hour conditioned medium were identified using the RayBio Human Growth Factor Array 1 and Human Inflammation Antibody Array 3 (RayBiotech, Inc., USA). Briefly, membranes containing immobilized antibodies against a select panel of inflammatory cytokines were incubated with conditioned media from the various cocultures overnight at 4°C with gentle agitation. The membranes were rinsed in wash buffer; then, biotin-conjugated secondary antibody cocktail was applied and the membranes were incubated for 2 hours at room temperature. Unbound antibodies were rinsed off, and the membranes were then incubated with HRP-Streptavidin for 2 hours at room temperature. The membranes were rinsed again, and subjected to chemiluminescence detection. Signal intensities were quantified using ImageJ (NIH, USA).

2.27. Statistical Analysis

Statistical differences were determined using a two-tailed Mann-Whitney U test. A p-value of < 0.05 was considered statistically significant.

**Chapter 3: Targeting of nuclear hormone
receptors in cancer-associated fibroblasts as an
adjuvant chemotherapy for squamous cell
carcinomas**

3.1. Introduction

Cancer development and progression are accompanied by the accrual of a reactive stroma (Gascard and Tlsty, 2016; Kalluri and Zeisberg, 2006). Far from being a passive bystander in the progression of disease, the reactive stroma fortifies tumor malignancy, modifies therapy outcomes and worsens patient prognosis (Gascard and Tlsty, 2016; Holohan et al., 2013; Kalluri, 2016; Kalluri and Zeisberg, 2006; Mezawa and Orimo, 2016). This occurs via a complex and dynamic interplay between tumor epithelia and stromal cells that is mediated by paracrine factors released into the tumor microenvironment. Tumor-stroma co-evolution further disrupts tissue organization (Lisanti et al., 2010; Martinez-Outschoorn et al., 2010), and the resultant loss of organ homeostasis creates a feed-forward reaction that is permissive to tumor growth and metastasis (Bizzarri and Cucina, 2014; Sonnenschein and Soto, 2016).

CAFs are the most abundant cell population in the tumor stroma of many solid cancers and interact intimately with tumor epithelia (Gascard and Tlsty, 2016; Kalluri, 2016). The origins of CAFs remain unclear, although local fibroblasts, bone marrow-derived mesenchymal stem cells, pericytes, and epithelial- and endothelial-mesenchymal transitioned cells contribute to the CAF population within tumors (Kalluri, 2016). CAFs are generally identified by their expression of alpha-smooth muscle actin (α -SMA) (Darby et al., 2016), fibroblast activation protein (FAP) (Santos et al., 2009), platelet-derived growth factor receptors (PDGFRs) (Paulsson et al., 2014), asporin (Simkova et al., 2016) and collagen 11 α 1 (Vazquez-Villa et al., 2015).

Functionally, CAFs are a potent source of growth factors, extracellular matrix (ECM) components, matrix remodeling enzymes, inflammatory cytokines and reactive oxygen species (ROS), all of which exacerbate cancer cell proliferation, invasiveness, apoptosis and energy metabolism, among other hallmarks of cancer (Arcucci et al.,

2016; Hanahan and Weinberg, 2011; Mezawa and Orimo, 2016). Despite the profound implications of CAFs for cancer progression, most of the approved cancer therapies are directed at the tumor epithelia rather than CAFs. The diversity of patient outcomes suggests that the rapid acquisition of resistance occurs and highlights that our understanding of CAF-facilitated oncogenesis is incomplete (Alderton, 2015a, b; Seton-Rogers, 2016; Zuccala, 2016). The use of combinatorial therapy that complements a cytotoxic drug with an adjuvant could subvert the acquisition of resistance.

The underlying transcription-based networks that enable the pro-oncogenic functions of CAFs remain poorly understood. Although transcription factor signaling nodes control many intracellular and extracellular signaling axes, transcription factors themselves are considered poor pharmacological targets; the activity of a large majority of transcription factors cannot be directly modulated by chemical drugs (Redmond and Carroll, 2009; Yan and Higgins, 2013). This is in contrast with kinases which can be easily switched on or off by small molecule compounds (Caunt et al., 2015; Thorpe et al., 2015). However, nuclear hormone receptors (NRs) represent a unique class of transcription factors that regulate gene expression under the strict control of endogenous or synthetic ligands (Bhagwat and Vakoc, 2015; Yin and Smith, 2016). There are a total of 48 NRs in humans and they play numerous roles in development, physiology and pathology. The binding of cognate ligands to NRs recruits co-activators or co-repressors that promote or suppress target gene expression. Thus far, the expression and function of NRs in CAFs are rarely considered outside the field of sex-steroid-responsive tumors such as those of the breast and prostate (Knower et al., 2013; Leach et al., 2015). Even then, the clinical relevance of the complete range of expressed NRs has not been explored.

In our study, we defined an NR profile for CAFs from patients diagnosed with cutaneous squamous cell carcinoma (SCC), the second-most common non-melanoma skin cancer (NMSC) after basal cell carcinoma, with a higher potential for local invasiveness and distal metastasis. Guided by this expression profile, the genetic and pharmacological targeting of specific driver NRs diminished several aggressive SCC hallmarks, namely, invasiveness, proliferation, drug resistance, energy metabolism and oxidative stress status. Furthermore, a combination treatment with NR ligands and cisplatin attenuated primary xenograft tumor growth and prevented the development of chemoresistance. Our findings suggest that NR-directed ligands that have successfully been used to treated other pathologies such as inflammation, dyslipidemia and diabetes, may be repurposed as adjunctive treatments to conventional anticancer chemotherapeutics.

3.2. Results

3.2.1. Nuclear receptors are differentially expressed between CAFs and normal fibroblasts

The complete NR profile of CAFs derived from cutaneous SCCs is currently unknown. Hence, paired samples of CAFs and peri-tumoral normal fibroblasts (FIBs) from archived SCC biopsies (n = 10) were obtained by laser capture microdissection (LCM) (Figure 6a), and their RNA was extracted and profiled for their expression of NR transcripts.

Among the 26 NR transcripts detected, 18 were upregulated (>2-fold), 5 remained unchanged and 3 were downregulated (>2-fold) in CAFs compared with normal FIBs (Figure 6b, left), regardless of whether the biopsy was taken from patients with metastatic or non-metastatic SCC tumors. Among the 3 NRs that were downregulated in CAFs, PPAR β expression was downregulated to the greatest extent, followed by VDR and GR.

Interestingly, both RXR α and RXR β were upregulated in CAFs. Because RXRs are promiscuous and form heterodimeric partners with many other NRs, their upregulation may represent an enabling characteristic of CAFs. Hence, we stratified the 21 differentially-expressed NRs according to whether they heterodimerized with RXRs and according to their ligand profiles. Nine of the 21 differentially expressed NRs were heterodimeric partners of RXRs (excluding the RXRs themselves) (Figure 6b, left). Thirteen of the 21 differentially expressed NRs had established ligand profiles and are thus targetable using available drugs.

For further *in vitro* study of CAFs, it was important to confirm whether the NR signature of SCC CAFs remains stable when CAFs are removed from their native environment for use in cell culture. Hence, we repeated the NR profiling with patient

CAFs that were explanted from human SCC tumors and cultured *in vitro* for 3-5 passages. A highly similar NR profile was observed between microdissected CAFs and the explanted CAFs (Figure 6b, right), indicating that the NR profile of SCC CAFs is retained during *in vitro* culture.

Figure 6. NRs are differentially expressed between CAFs and FIBs. (a) Representative images of H&E-stained sections of archived patient SCC biopsies before (left) and after (right) LCM. CAFs within the tumor stroma directly bordering tumor epithelia were selectively excised while excluding obvious immune or vascular structures. Scale bar: 1 cm. **(b)** The relative expression of detectable NR transcripts in CAFs obtained from metastatic and non-metastatic patient SCC biopsies (n=10), compared with the expression in matched pairs of FIBs using RT-qPCR (left). Profiling was repeated on CAFs explanted from human SCC tumors (n=5) and

cultured *in vitro* (right). NRs that form heterodimers with retinoid X receptors (RXR) are labelled in red, while those that form homodimers are labelled in blue. Numbers in superscript distinguish liganded NRs (1) from orphan NRs (2). Color scales: Green = downregulated, red = upregulated. “N.D.” denotes that the gene was not detected in the RT-qPCR experiment.

3.2.2. The SCC transcriptome is modified by changes in the CAF NR profile

We hypothesized that by altering the NR status in CAFs, the paracrine signaling network between cancer cells and CAFs will be disrupted, resulting in changes to the cancer cell transcriptome that may undermine the hallmarks of cancer. Guided by the NR profile of SCC CAFs, the individual knockdown or overexpression of specific NRs in CAFs was performed. Briefly, if the expression of an NR was downregulated in CAFs relative to FIBs, that NR was overexpressed (NR-OE). Conversely, an NR that was highly expressed in CAFs relative to FIBs was subjected to siRNA knockdown (NR-KD). NR-OE or NR-KD status was verified using RT-qPCR (Figures 7 and 8). NR-OE/KD-CAFs were subsequently co-cultured with the malignant SCC cell line A-5RT3 cells for 48 hours. The A-5RT3 cells were then isolated, and their RNA was extracted and processed for use in microarray (Figure 7 and 8).

Figure 7. Design of the experiments based on the NR profile of CAFs. After NR profiling, the genetic overexpression or knockdown of specific NRs was performed in CAFs, followed by their co-culture with the malignant SCC cell line A-5RT3. A-5RT3 cells were subsequently isolated for microarray analysis or subjected to functional assays to evaluate the non-cell autonomous effects of NR-KD/OE-CAF s on malignant phenotypes.

Figure 8. RT-qPCR validation of NR overexpression or knockdown in CAFs. The ribosomal RNA gene Rpl27 was used as a housekeeping gene. NRs shown in the box belong to Cluster A of microarray analysis. The values shown are means $\pm$ S.D. from $n=3$ independent experiments. * $p < 0.05$, ** $p < 0.01$.

Microarray data analysis showed that 302 genes were differentially expressed by >1.5-fold between the A-5RT3 cells from A-5RT3:CAF co-culture and A-5RT3:FIB co-culture (Figure 9a). Using the Ingenuity Pathway Analysis software to further dissect the microarray data obtained from the A-5RT3 cells, under the ontology term “Tumor morphology” was enriched ($p < 0.024$) and “Cancer” was appropriately identified as the most-enriched pathology ($p < 0.030$). Next, we identified molecular and cellular functions in A-5RT3 cells that were altered by co-culture with CAFs. “Cellular movement” ($p < 0.029$), “Cell morphology” ($p < 0.024$), “Cellular Function and Maintenance” ($p < 0.024$), “Cellular Growth and Proliferation” ($p < 0.027$) and “Cell Cycle” ($p < 0.024$) were the most pertinent functions associated with this 302-member gene list (Figure 9b). Top networks in our analysis included “Organismal injury and abnormalities” (score = 37), “Cancer” (score = 34), “Cell death and survival” (score = 22), “Cell movement, signaling and interaction” (score = 18) and “Inflammation” (score = 15) (Figure 10). The network analysis shows that a wound-like environment is created when A-5RT3 cells are co-cultured with CAFs.

To understand how the individual knockdown or overexpression of NRs in CAFs affected the A-5RT3 transcriptome, the expression of the 302 genes was further examined under all A-5RT3:NR-KD/OE-CAFs co-culture conditions. Hierarchical clustering resulted in two major clusters: Cluster A (PPAR β / δ -OE-CAFs, AR-KD-CAFs, GR-OE-CAFs, RAR β -KD-CAFs, and VDR-OE-CAFs) and Cluster B (others). Cluster B contained two sub-clusters, B1 and B2. Cluster B2 gene signatures were similar to those of the A-5RT3:CAF co-culture, indicating a functional redundancy among these CAF NRs, i.e., perturbation of their expression has limited impact on cancer cell behavior.

Figure 9. The A-5RT3 cell transcriptome is modified by co-culture with FIBs, CAFs and NR-KD/OE-CAFs. Heatmap and clustering of the 302 genes that were differentially expressed by >1.5-fold between A-5RT3:CAF and A-5RT3:FIB co-culture samples. The same gene set was evaluated for all A-5RT3:NR-KD/OE-CAFs co-cultures. Colour scales: Green = downregulated; red = upregulated. Cluster A (yellow): NR-KD/OE-CAFs conditions that distinctly modify A-5RT3 gene expression, identified by the inverse regulation of 70 genes when compared with A-5RT3:CAF co-culture. **(b)** Gene ontologies enriched in the identified 302 gene set are ranked in decreasing order of the strength of association based on their p-value. A

higher degree of association is indicated by a smaller p-value. Only the top 5 ontologies with p-values <0.05 are included.

Although Cluster B1 gene signatures differ from the A-5RT3:CAF gene signature, pharmacological targeting of these NRs is difficult because they are orphan receptors. In contrast, Cluster A alterations to the NR status of CAFs clearly modified the transcriptome of A-5RT3 cells in co-culture, as evidenced by the inverse regulation of 70 genes (i.e. genes that were downregulated in A-5RT3 cells from Cluster A co-cultures as opposed to being upregulated in the A-5RT3:CAF co-culture) (Figure 9a). Importantly, genes associated with EMT and tumor malignancy such as HGF, ZEB1, POSTN and IL6 were strongly upregulated in the A-5RT3:CAF co-culture but attenuated in A-5RT3 cells that were co-cultured with Cluster A NR-KD/OE-CAFs (Figure 10). This finding suggests that individual NRs belonging to Cluster A have attenuating effects on CAF-dependent aggressive tumor phenotypes.

3.2.3. Genetic modulation of the NR expression of CAFs inhibits SCC malignancy and reduces chemoresistance

Based on the microarray analysis, SCC invasion, proliferation, drug resistance, energy and ROS metabolism were most likely to be altered by the CAF NR status, particularly, that of NRs belonging to Cluster A. Before beginning functional assays to evaluate the effect of these NR-KD/OE-CAFs on SCC phenotypes, we first confirmed the overexpression or knockdown of these candidate NRs at the protein level (Figure 11).

Figure 11. Immunoblots of PPARβ/δ, AR, GR, RARβ and VDR in FIBs, CAFs and NR-KD/OE-CAFs. β-Tubulin from the same samples was used as a loading and transfer control. The molecular weights of the proteins are indicated in kiloDaltons. The numbers below the immunoblots represent mean densitometry measurements from n=3 independent experiments.

Next, we examined the invasiveness of A-5RT3 cells using a Boyden chamber assay; FIBs, CAFs or NR-KD/OE-CAFs were seeded in the lower chamber to provide the chemotactic gradient. CAFs potentiated the invasiveness of A-5RT3 cells two-fold compared to FIBs. While RARβ-KD-CAFs did not significantly reduce A-5RT3 cell invasiveness, PPARβ/δ-OE-CAFs and VDR-OE-CAFs reduced A-5RT3 cell

invasiveness by approximately 50% and 75% respectively compared with A-5RT3:CAFs co-culture. AR-KD-CAFs and GR-OE-CAFs also inhibited A-5RT3 cell invasion by 27.5% and 23.0% respectively (Figure 12a).

Next, we examined the proliferation of A-5RT3 cells co-cultured with FIBs, CAFs and NR-KD/OE-CAFs. BrdU incubation followed by FACS analysis revealed that A-5RT3:CAFs co-cultures had increased proliferation when compared with A-5RT3:FIBs co-culture ($53.4 \pm 2.4\%$ vs. $33.5 \pm 5.1\%$ in the S-phase respectively) (Figure 12b). Except for GR-OE-CAFs, all other NR-KD/OE-CAFs inhibited A-5RT3 cell proliferation by $\geq 14\%$ (Figure 12b).

Although cisplatin is widely used in SCC chemotherapy, drug resistance arises quickly. Our microarray analysis and literature review suggested that chemoresistance is reinforced by the tumor stroma. CAF conditioned medium (CCM) conferred chemoprotective effects on A-5RT3 tumor cells that had been treated with cisplatin, as evidenced by a two-fold increase in the IC_{50} dose of cisplatin compared with A-5RT3 cells in FIB CM (FIB CM IC_{50} : $19.8 \mu\text{g/mL}$ vs CAF CM IC_{50} : $39.7 \mu\text{g/mL}$). IC_{50} is defined as the cisplatin dose required to reduce A-5RT3 cell survival to 50% of the total cell population. GR-OE-CAF CM increased the IC_{50} compared with CAF CM, whereas AR-KD-CAF CM had no significant effect on the IC_{50} . In contrast, VDR-OE-CAF CM abrogated CAF-mediated chemoresistance to the greatest extent, requiring only $11.2 \mu\text{g/mL}$ cisplatin to achieve IC_{50} , followed by PPAR β/δ -OE-CAF CM ($24.4 \mu\text{g/mL}$) and RAR β -KD-CAF CM ($27 \mu\text{g/mL}$) (Figure 12c).

Altered energy metabolism is a hallmark of cancer. The A-5RT3:CAF co-culture displayed a 60% increase in glucose uptake with a concomitant 40% elevation of adenylate energy charge when compared with the A-5RT3:FIB co-culture (Figure 12d-e). Although GR-OE-CAFs and RAR β -KD-CAFs did not significantly reduce A-

5RT3 glucose uptake and energy charge, PPAR β/δ -OE-, VDR-OE- and AR-KD-CAFs inhibited glucose uptake by up to 50% (Figure 12d) and, correspondingly, lowered the adenylate energy charge of the co-cultured A-5RT3 cells (Figure 12e).

Reactive oxygen species (ROS) mediate cellular signaling, and elevated intratumoral ROS is a known consequence of aberrant cancer cell metabolism that is exacerbated by the tumor stroma. Indeed, the A-5RT3:CAFs co-culture displayed a 40% increase in intracellular ROS production relative to the A-5RT3:FIBs co-culture. Only VDR-OE-CAFs were able to significantly reduce ROS production in A-5RT3 cells (Figure 12f)

To consolidate the efficacy of targeting specific CAF NRs for the inhibition of SCC hallmarks, we overlaid the multi-parametric data from A-5RT3:NR-KD/OE-CAF co-cultures onto the data from A-5RT3:CAF and A-5RT3:FIB co-cultures (Figure 13). This allows easy visualization of whether an NR in CAFs could simultaneously inhibit two or more phenotypes of aggressive SCC. We observed that CAFs potentiated all six aggressive behaviors of the co-cultured A-5RT3 cells (red line) compared with FIBs (green line). Attenuation of any of these six SCC behaviors by NR-KD/OE-CAFs is indicated when the black line approaches the green line. Our analysis suggests that the expression of PPAR β/δ , VDR and, to a lesser extent, AR and RAR β in CAFs elicits antitumor effects and reduces chemoresistance (Figure 13).

Figure 12. Genetic modulation of CAF NR expression inhibits SCC malignancy and reduces chemoresistance. (a) Relative A-5RT3 invasiveness when co-cultured with FIB, CAF or NR-KD/OE-CAFs. (b) The percentage of A-5RT3 cells in the S-phase of the cell cycle when co-cultured with FIBs, CAFs or NR-KD/OE-CAFs is identified by the incorporation of BrdU into DNA. (c) Dose-response curves for apoptosis of A-5RT3 cells cultured in FIB, CAF or NR-KD/OE-CAF conditioned medium and subjected to the indicated concentrations of cisplatin for 16 hrs. IC₅₀ was calculated using the graphing software, Prism. (d) Relative glucose uptake by A-5RT3 cells that were co-cultured with FIB, CAF or NR-KD/OE-CAFs for 48 hrs. (e) Relative adenylate energy charge of A-5RT3 cells that were co-cultured with FIB, CAF or NR-KD/OE-CAFs for 48 hours. (f) Relative intracellular ROS production by A-5RT3 cells co-cultured with FIB, CAF or NR-KD/OE-CAFs for 48 hours. Values represent means $\pm$ S.D. from n=3 independent experiments. * $p < 0.05$, ** $p < 0.01$

Figure 13. Multi-parametric evaluation of the effect of CAF NRs on SCC. The measured parameters from A-5RT3 cells that were co-cultured with FIB (green line), CAF (red line) and NR-KD/OE-CAFs (black line) are superimposed. Shading represents the standard error of each parameter. A-5RT3 parameters that were significantly altered by co-culture with NR-KD/OE-CAFs are boxed in green.

Finally, the CM from the co-cultures was subjected to a multiple analyte detection assay that screened for 16 secreted pro-tumor factors. Our results show that

the paracrine signaling network between the CAFs and SCC cells was indeed disrupted by the altered NR status of the CAFs (Figure 14). In particular, HGF and VEGF-C levels in the CM were attenuated to the greatest extent by NR-KD/OE-CAFs.

Figure 14. Heatmap showing the relative quantities of analytes detected in the conditioned medium of A-5RT3:FIBs, A-5RT3:CAFs or A-5RT3:NR-KD/OE-CAF co-cultures. Fold differences are relative to A-5RT3:FIB conditioned medium. Color scales: Green = downregulated; red = upregulated.

Collectively, these results demonstrate that the transcriptome of SCC cancer cells is sensitive to the NR status of CAFs and suggest that the NR status of CAFs inhibits SCC hallmarks by modulating tumor-stroma crosstalk.

3.2.4. Pharmacological modulation of CAFs NRs inhibits SCC malignancy and reduces chemoresistance *in vitro*

The overexpression or knockdown of specific NRs in CAFs resulted in the simultaneous attenuation of several cancer hallmarks. To test the translatability of CAF NR-directed therapy into an experimental clinical setting, we selected small molecule agonists of PPAR β/δ (GW0742), VDR (EB1089) and GR (fluticasone propionate), or antagonists of RAR β (LE135) and AR (bicalutamide) for testing. We first assessed whether these ligands exhibited any intrinsic anticancer activity. A-5RT3 cells that were treated with 5 μ M of each ligand for 24 hours and analyzed by FACS for BrdU uptake and Annexin-PI staining; the results revealed that only EB1089, the VDR agonist, significantly reduced A-5RT3 cell proliferation and increased A-5RT3 cell apoptosis (Figure 15a, b).

To explore whether these NR ligands exert anticancer effects via CAFs, we pre-treated CAFs with the ligands for 48 hours. The CAFs were then rinsed thoroughly to remove excess ligand, and fresh serum-free medium was added for conditioning. The 48-hour CM from pre-treated CAFs was then collected and added to A-5RT3 cells. CAFs CM potentiated A-5RT3 cell proliferation compared with FIBs CM. CM from GW0742 and fluticasone propionate pre-treated CAFs did not significantly alter A-5RT3 cell proliferation. Interestingly, A-5RT3 cells cultured in LE135 or bicalutamide pre-treated CAFs CM exhibited reduced proliferation (Figure 15c). This result was not observed when A-5RT3 cells were directly exposed to the ligands, suggesting that the effect was CAF-dependent. In contrast, CM from EB1089 pre-treated CAFs did not have any anti-proliferative effect, indicating that EB1089 must act directly on A-5RT3 cells to inhibit their proliferation (Figure 15c).

Figure 15. Pharmacological modulation of CAF NRs inhibits SCC malignancy and reduces chemoresistance *in vitro*. (a) Percentages of A-5RT3 cells in the G0/G1-, S- or G2-phases of the cell cycle when subjected to various NR ligand treatments. (b) Percentages of A-5RT3 cells that stained positive for Annexin-V when subjected to the indicated treatments. (c) Percentages of A-5RT3 cells in the G0/G1-, S- or G2-phases of the cell cycle. A-5RT3 cells were cultured in the conditioned medium of CAFs that had been subjected to NR ligand treatments. (d) Percentages of A-5RT3 cells that stained positive for Annexin-V. Cisplatin (10 µg/mL) was added to A-5RT3

cells that were cultured in the conditioned medium of CAFs that had been subjected to various NR ligand treatments. **(e)** Relative mRNA expression of the indicated NRs in FIBs, CAFs and A-5RT3 cells. The ribosomal RNA gene Rpl27 was used as a housekeeping gene. The values represent the means $\pm$ S.D. from n=3 independent experiments. n.s.: not significant, * $p<0.05$, ** $p<0.01$.

Based on the above observations, we hypothesized that NR ligands may be more suitable as an adjunctive therapy rather than as a primary anticancer treatment. Therefore, we asked whether our selected NR ligands could sensitize A-5RT3 cells to cisplatin. A-5RT3 cells were cultured in pre-treated CCM in the presence of 10 μ g/mL cisplatin. Nearly 40% of A-5RT3 cells underwent apoptosis when cultured in FIB CM, compared with only 5% when cultured in CCM. Pre-treatment of CAFs with LE135, bicalutamide and EB1089 abrogated the CAF-enabled resistant phenotype in A-5RT3 cells (Figure 15d).

In the earlier NR-KD/OE-CAF experiments, VDR and PPAR β/δ exhibited potent anticancer properties. However, pharmacologically, RAR β and AR appeared to be better targets for CAF-directed therapy. Therefore, we explored whether this discrepancy could be explained by the relative expression of the NRs in A-5RT3 cells and CAFs. qPCR analyses revealed that VDR expression was higher in the A-5RT3 cells than CAFs (Figure 4e), suggesting that A-5RT3 cells are more responsive to EB1089. Unlike CAF-VDR-OE, which demonstrated anticancer effects, the pretreatment of CAFs with EB1089 was of limited use because of the low endogenous expression of VDR. Conversely, RAR β and AR expression was much higher in the CAFs than A-5RT3 cells (Figure 15e). Knockdown of RAR β and AR in CAFs inhibited some aggressive SCC phenotypes. Similarly, RAR β and AR antagonists that bind to the abundant levels of their cognate NRs in the CAFs were able to recapitulate the effects of RAR β /AR-KD-CAF (Figure 13, 15c-d). Collectively, our *in vitro* data

suggests that LE135 and bicalutamide (and thus RAR β and AR) are good NR candidates for CAF-directed adjunct chemotherapy.

3.2.5. Cisplatin with LE135 and bicalutamide adjunct therapy inhibits SCC malignancy and reduces chemoresistance in vivo

Based on our *in vitro* observations, combinatorial treatment using cisplatin with LE135 and/or bicalutamide as an adjunct therapy could be effective against SCC. To test the efficacy of such treatments *in vivo*, we first titrated the dose of cisplatin for use in immunodeficient NSG mice bearing subcutaneous A-5RT3:CAF xenografts. A minimal intraperitoneal dose of 2 mg/kg cisplatin (administered twice on weeks 3 and 4) was required to impede tumor growth (Figure 16a). Although 10 mg/kg cisplatin reduced tumor volume, this dose was accompanied by proteinuria (Figure 16a, b) indicating nephrotoxicity, a common side effect of cisplatin chemotherapy. Therefore, a dose of 2 mg/kg cisplatin in combination with 5 mg/kg LE135 (Hong et al., 2014; van de Pavert et al., 2009) and 10 mg/kg bicalutamide (Tesei et al., 2013; Thomas et al., 2013), administered twice (at weeks 3 and 4).

Figure 16. Tumor volume and proteinuria measurements in mice receiving intraperitoneal cisplatin. (a) Tumor volumes of NSG mice (n=4 per experimental condition, 2 tumors per mouse) bearing A-5RT3:CAF tumor xenografts treated with the indicated cisplatin doses, which were administered by intraperitoneal injection. Representative images of excised tumors are shown on the right. (b) Proteinuria measurements obtained from the urine of mice treated with the indicated cisplatin doses.

Cisplatin with vehicle (CV) treatment resulted in slower tumor growth. However, tumor volume continued to increase beyond week 4. Cisplatin with either LE135 (CL) or bicalutamide (CB) treatment displayed stronger, albeit insignificant, inhibition of tumor growth. Only treatment with cisplatin combined with both LE135 and bicalutamide (CBL) resulted in a significant delay of tumor growth, although tumor remission, defined as a reduction in tumor volume, was not achieved (Figure 17a). Tumor histology revealed that Laminin 5 expression, which is inversely associated with tumor invasion potential, was only restored by CBL treatment (Figure 17b). The expression of the epithelial marker E-cadherin (E-cad) became stronger and more defined at cell:cell boundaries with CL, CB and CBL treatments compared with CV treatment. The expression of the proliferation marker Ki67 was reduced by CL, CB and CBL treatments compared with CV treatment. TUNEL assay further showed that the number of apoptotic tumor cells was substantially increased only by CBL therapy. However, staining for cisplatin adducts revealed weak accumulation of cisplatin within the tumors across all treatments (Figure 17b).

Similar observations were made using immunoblot analyses of tumor lysates, confirming that the properties of A-5RT3:CAF tumor xenografts were altered by the indicated treatments. MMP2, PCNA and GLUT1 expression was significantly reduced only by CBL therapy, suggesting that the complete combination of drugs was required

to impair tumor invasiveness, proliferation and energy metabolism respectively. The expression of caspase 3 was strongly elevated by CL and CBL treatment (Figure 18).

Figure 17. Cisplatin with LE135 and bicalutamide adjunct therapy inhibits SCC malignancy and reduces chemoresistance *in vivo*. (a) Tumor volume measurements in mice implanted with A-5RT3:CAF xenograft tumors (n =4 per experimental condition, 2 tumors per mouse) receiving intraperitoneal injections of 2 mg/kg cisplatin, alone (CV) or in combination with 5 mg/kg LE135 (CL), 10 mg/kg bicalutamide (CB), or both (CBL). Treatments were administered on two consecutive weeks (d21 and d28, treatment: "on"). (b) Immunofluorescence staining for pan-cytokeratin (PanCK), laminin 5 (Lam 5), E-cadherin (E-cad), Ki67, TUNEL and cisplatin adducts in xenograft tumors of mice receiving the indicated treatments. Scale bar: 100 μ m.

Figure 18. Immunoblots and densitometry measurements of MMP2, PCNA, caspase 3 and GLUT1 in mice receiving the indicated treatments. β -Tubulin from the same samples was used as a loading and transfer control. Target protein molecular weights are shown in kilodaltons. The bar chart shows normalized densitometry measurements of the immunoblots. The values shown represent means $\pm$ S.D. from n=3 independent experiments. * $p<0.05$, ** $p<0.01$.

These results suggest that the attenuation of tumor aggressiveness was possible via the intraperitoneal injection of NR ligands, but tumor remission did not occur due to insufficient intratumoral accumulation of the cytotoxic agent, cisplatin. Hence, delivering cisplatin directly into the tumor could improve treatment outcomes. Because SCC tumors are located superficially, intratumoral injection of cisplatin is viable. Importantly, the inflection point at week 4 in the tumor growth curve suggests that cisplatin resistance was already developing (Figure 5a).

To confirm the efficacy of an intratumoral injection of cisplatin, we began treatment of another set of tumor xenografts in NSG mice. On weeks 3 and 4 when xenograft volumes approached 150 mm³, 2 mg/kg cisplatin was injected directly into the tumors while 5 mg/kg LE135 and/or 10 mg/kg bicalutamide remained as intraperitoneal injections, given their effect in earlier experiments. CL, CB and CBL treatments resulted in significantly greater tumor remission compared to CV treatment, suggesting that the combined therapy had sensitized the tumors to cisplatin. Xenografts on mice that received CL, CB and CBL treatment became practically impalpable by week 6, even though treatment was discontinued.

To observe whether tumors would recur, the same mice were maintained for several weeks after the discontinuation of treatments (Figure 19a). Tumors reappeared at week 7 for mice that received the CV, CL and CB therapies. Tumor recurrence was also seen in CBL-treated mice at week 9, indicating the presence of residual disease that had not been eliminated by the first round of combinatorial therapy. This presented an opportunity to study whether the surviving A-5RT3:CAF tumors had developed resistance. Tumors were allowed to grow until tumor volumes approached 150 mm³, at which time treatment was resumed (the mice were treated on weeks 11 and 12) . CV-, CL- and CB-treated tumor xenografts continued to increase in size. However, we noted that CL treatment delayed tumor growth (Figure 19a).

CBL therapy was started at the same time as the other treatments despite the tumors being smaller at week 11. If the tumor had gained resistance, tumor volume would have reached a plateau or increased during the second round of treatment. Instead, CBL treated tumors responded optimally and shrank until they became barely palpable (Figure 19a).

Figure 19. Intratumoral injection of cisplatin increases the efficacy of combinatorial therapy. (a) Tumor volume measurements in mice implanted with A-5RT3:CAF xenograft tumors (n=4 per experimental condition, 2 tumors per mouse) receiving intratumoral injections of 2 mg/kg cisplatin, alone (CV) or in combination with intraperitoneal injections of 5 mg/kg LE135 (CL), or 10 mg/kg bicalutamide (CB), or both (CBL). Treatments were administered on weeks 3 and 4, withdrawn from weeks 5 to 10 and resumed on weeks 11 and 12. **(b)** Immunofluorescence staining for pan-cytokeratin (PanCK), laminin 5 (Lam 5), E-cadherin (E-cad), Ki67, TUNEL and cisplatin adducts in xenograft tumors of mice receiving the indicated treatments. Scale bar: 100 μ m.

Immunofluorescence staining showed that E-cadherin expression and localization was partially restored by CL and CB treatments as compared to CV. CL treatment resulted in a slight increase in the number of apoptotic cells and intratumoral cisplatin accumulation based on TUNEL assay and staining for cisplatin-DNA adducts. CBL therapy was most effective in restoring laminin 5 expression and was accompanied by intense and well-defined E-cad staining; simultaneously, Ki67 staining was reduced and the number of TUNEL-positive nuclei increased (Figure 19b). Importantly, the numerous cisplatin adducts in CBL-treated tumors suggest that CBL therapy plays a role in preventing chemoresistance. These findings were validated by immunoblot analyses of tumor lysates which revealed that MMP9 expression was strongly repressed by CL and CBL treatment. PCNA and GLUT1 expression was reduced only by CBL treatment. Caspase 3 protein expression was upregulated 4-fold by CL treatment and 3-fold by CB treatment. CBL therapy was the most potent, causing a >100-fold increase in caspase 3 protein expression (Figure 19b).

Finally, immunoblot (MMP, PCNA, GLUT1 and caspase 3) and immunofluorescence (cisplatin adduct) data representing SCC tumor invasiveness, proliferation, energy metabolism, apoptosis and cisplatin sensitivity respectively, were

consolidated into a multi-parametric chart of treatment efficacy. The red line represents the tumor response to CV treatment which forms the baseline for comparison. The green line represents tumor response to CL, CB or CBL therapy. Our data suggests that, guided by our initial NR profiling of CAFs, which identified RAR β and AR as important mediators of malignancy, the antagonism of both of those NRs alongside cisplatin chemotherapy most effectively inhibits aggressive SCC hallmarks and increases the potency of cisplatin while preventing chemoresistance.

Figure 20. Immunoblots and densitometry measurements for MMPs, PCNA, caspase 3 and GLUT1 in mice receiving the indicated treatments. β -Tubulin from the same samples was used as a loading and transfer control. Target protein molecular weights are shown in kilodaltons. The bar chart shows normalized densitometry measurements of the immunoblots. The values shown represent the means $\pm$ S.D. from $n=3$ independent experiments. * $p<0.05$, ** $p<0.01$.

Figure 21. Multi-parametric evaluation of the effect of CAF NRs on SCC *in vivo*. The measured parameters for the CV-treated tumors (red line) and combinatorial therapies (CL, CB, CBL; green line) are superimposed. The shading represents the standard error of each parameter. Parameters that are significantly altered by co-culture with NR-KD/OE-CAFS are boxed in green.

3.3. Conclusions

In this chapter, we demonstrated that CAFs from SCC tumors have a unique NR signature that is preserved even when they are explanted from the tumor microenvironment for *in vitro* culture. Based on this NR signature, the genetic overexpression or knockdown of specific NRs in CAFs caused global changes to the SCC transcriptome, resulting in the non-cell autonomous inhibition of multiple SCC hallmarks. The secreted protein levels of mitogenic and pro-metastatic factors in the extracellular microenvironment was also reduced. Furthermore, the antagonists to RAR β and AR, when used in combination with cisplatin, effected potent anti-tumor outcomes *in vitro* and *in vivo*. In addition to the attenuation of tumor invasiveness, proliferation and glycolytic metabolism, there was a sustained intratumor accumulation of DNA adducts corresponding with increased apoptosis in tumors treated with combination therapy that did not occur when cisplatin was administered alone. Our data show that the NR profiling of CAFs can facilitate the rational selection of NR ligands to limit chemoresistance, thereby extending the clinical efficacy of traditional chemotherapy drugs.

Chapter 4: Cancer-associated fibroblasts enact field cancerization by promoting extratumoral oxidative stress

4.1. Introduction

Tumor initiation, growth and propagation are frequently accompanied by desmoplasia and the acquisition of a reactive stroma, which comprises CAFs (Tlsty and Hein, 2001; Udagawa and Wood, 2010). The dynamic and reciprocal relationship between the epithelial and mesenchymal compartments of the tumor dictates almost every aspect of cancer progression, even governing the efficacy of therapy and influencing the risk of disease relapse (Olumi et al., 1999; Shimoda et al., 2010). Importantly, a population of epithelial and stromal cells adjacent to the primary neoplastic lesion acquires early genetic changes but lacks histopathology, indicating that tumors prime the surrounding tissue for malignancy, an effect known as field cancerization (Braakhuis et al., 2003; Rivenbark and Coleman, 2012). Field cancerization results in the appearance of multifocal primary tumors in close proximity with a higher chance of recurrence after the surgical resection of malignant tumors (Dotto, 2014). Although genetic and epigenetic anomalies have been well described as the driving force behind the field cancerization of premalignant epithelia, mediators of field cancerization by the stroma remain poorly understood.

The expansion of a cancer field requires soluble factors that promote oncogenic transformation in a non-cell-autonomous manner and prime the adjacent naïve stroma to support tumor growth. Although these functions have usually been ascribed to growth factors and cytokines, ROS which are persistently elevated in almost all cancers, have been recently identified as critical intermediates of cellular signaling (Chaiswing and Oberley, 2010; Liou and Storz, 2010; Lisanti et al., 2010). The oxidative modification of proteins by ROS can modulate intracellular signaling to influence cellular behavior and contribute to the pathophysiology of many human diseases (Ray et al., 2012; Zhang et al., 2011). ROS are by-products of aberrant

metabolism in the tumor epithelia, and their cell-autonomous mutagenic effects have been studied extensively. Much less is known about the regulation of ROS in CAFs and how ROS impact field cancerization. Notably, not all ROS are well suited for paracrine cell signaling. Superoxide anions ($O_2^{\cdot-}$) and hydroxyl radicals ($OH\bullet$) have a very short diffusion distance because they are highly reactive, resulting in a very short half-life in the aqueous phase (Baker and Gebicki, 1984). These ROS also have a very low permeability coefficient for lipid bilayers (Takahashi and Asada, 1983). In contrast, H_2O_2 has a longer half-life, is lipid soluble and can diffuse across the cellular plasma membrane, thereby making it an ideal field effect carcinogen. In this study, we investigated the regulation of ROS, and in particular, H_2O_2 production, in CAFs and examined how this tumor stroma-mediated oxidative stress causes a premalignant field defect.

4.2. Results

4.2.1. ROS are elevated in the microenvironment of numerous tumor types

We first examined the oxidative status of tumor sections from patients with late stage (stage 3 to metastatic) SCC, as well as gastric, breast and colon cancers using the ROS-sensitive fluorescent dye CM-H₂DCFDA. Pan-cytokeratin staining was performed to distinguish between tumor epithelia and stromal elements. All tumor sections stained positive for ROS (Figure 22a). SCC sections exhibited the highest intensity of ROS staining. Interestingly, ROS were not restricted to the tumor epithelia but were frequently observed in the stromal compartments of tumors. To identify the cell types responsible for the elevated microenvironmental ROS levels, we measured extracellular H₂O₂ in conditioned medium from various SCC lines of different aggressiveness (Figure 22b). HaCaT is a non-tumorigenic human keratinocyte cell line, while the benign A-5, malignant II-4 and highly aggressive A-5RT3 lines are sublines of different tumorigenic potential that are derived from HaCaT cells (Boukamp et al., 1999). HaCaT cells secreted the lowest amount of H₂O₂, while conditioned medium from A-5RT3 cells had the highest level of H₂O₂ (Figure 22b). Because CAFs dominate the tumor stroma and diffusible H₂O₂ is well suited for a role in tumor-stroma crosstalk, we measured the amount of H₂O₂ in conditioned medium from CAFs that were explanted from four patient skin SCC biopsies alongside their paired normal fibroblasts (FIBs) (Figure 22c). We consistently detected higher extracellular H₂O₂ levels in the conditioned medium from SCC CAFs compared to that of FIBs.

Figure 22. Elevated ROS levels in the microenvironment of numerous tumor types. (a) Dichlorofluorescein diacetate (DCF) staining to detect ROS on sections of patient tumor biopsies obtained from the skin, gastric, breast and colon. Pan-cytokeratin (CK) antibody staining was used to delineate epithelial-stromal boundaries. The sections were counterstained with DAPI. Scale bar: 100 μm . B-C, Extracellular concentrations of H_2O_2 in the conditioned medium from (b) various human SCC lines, and (c) paired FIBs and CAFs obtained from human SCC explants, as measured using the Amplex Red H_2O_2 assay. * $p < 0.05$, ** $p < 0.01$.

4.2.2. Microenvironment oxidative stress causes a premalignant field defect

Next, we wanted to determine whether microenvironmental oxidative stress promotes premalignant development, i.e., field cancerization. Because the tumor promotion protocol involves the use of a mutagen, MNNG, we first determined the cytotoxic dose of MNNG in combination with various concentrations of H_2O_2 . We did not observe any significant differences in the percentages of apoptotic (Annexin V^{high}/PI^{low} and

Annexin V^{high}/PI^{high}) and necrotic (AnnexinV^{low}/PI^{high}) keratinocytes treated with 1-100 nM H₂O₂ alone or in combination with 10 ng/mL MNNG (Tables 5 & 6). Thus, these ranges of H₂O₂ and MNNG were used to examine the effects of H₂O₂ on colony formation in soft agar. A marked increase in colony number was observed with cells that had been pretreated with MNNG and exposed to H₂O₂ for 4 weeks (Figure 23a).

MNNG \ H ₂ O ₂	0 nM	10 nM	100 nM	1 μM	10 μM	100 μM
0 ng/ml	6.0	6.8	5.9	4.7	3.8	6.1
10 ng/ml	5.6	5.7	3.5	3.7	2.6	4.3
0.1 μg/ml	3.4	9.6	9.5	5.1	5.3	3.9
1 μg/ml	8.8	11.9	12.4	11.8	15.2	14.4

Table 5. Percentage of apoptotic cells after exposure to the indicated concentrations of MNNG and H₂O₂. Apoptotic cells were determined by FACS analysis (10,000 events). Sum of Annexin V_{high}/PI_{low} (early apoptosis) and Annexin V_{high}/PI_{high} (late apoptosis) were considered apoptotic. Values (bold) denote significant increased apoptotic cells (%) when compared with untreated keratinocytes. Values were means of three independent experiments. The data were analyzed using FlowJo software.

MNNG \ H ₂ O ₂	0 nM	10 nM	100 nM	1 μM	10 μM	100 μM
0 ng/ml	5.6	3.8	5.0	5.6	7.2	5.6
10 ng/ml	8.5	9.2	9.5	11.7	13.3	14.5
0.1 μg/ml	10.8	11.0	12.6	14.5	14.1	14.9
1 μg/ml	14.4	12.7	13.2	16.1	18.2	21.9

Table 6. Percentage of necrotic cells after exposure to indicated concentration of MNNG and H₂O₂. Necrotic cells (Annexin V_{low}/PI_{high}) were determined by FACS analysis (10,000 events). The values (in bold) denote significant increase in necrotic cells (%) when compared with untreated keratinocytes. The values shown represent the means of three independent experiments. Data were analyzed using FlowJo software.

Keratinocytes treated with a higher concentration of H₂O₂ alone formed colonies in soft agar, suggesting that chronic exposure to elevated extracellular H₂O₂ facilitates the transformation of epithelial cells. Normal and neoplastic epithelial cells may respond differently to H₂O₂. Indeed, we observed differential growth response

and invasiveness to exogenous H_2O_2 . The proliferation of HaCaT and A-5RT3 cells peaked at 10 nM H_2O_2 , while the proliferation of A-5 and II-4 cells peaked at 100 nM H_2O_2 .

Figure 23. H_2O_2 has a transforming effect on epithelia *in vitro*. (a) Quantification of epithelial colonies in soft agar at 1 month after the indicated treatments with MNNG and H_2O_2 . (b) Percentages of proliferating (S-phase) cells after the indicated treatments with H_2O_2 as determined by BrdU labeling and FACS analysis. (c) Relative fluorescence (SYTO60 dye) of invaded cells after the indicated treatments in a transwell invasion assay. (d) FACS analysis of the epithelial cell lines subjected to the indicated concentrations of H_2O_2 treatments for 24 h, then stained using Annexin-V FITC antibodies and propidium iodide to identify apoptotic cells. The data are presented as the mean $\pm$ S.D. of 3 independent experiments. Statistical tests were

performed against cognate untreated cells for each cell type. n.s. not significant, * $p < 0.05$, ** $p < 0.01$.

The proliferation of HaCaT cells began to decrease as the concentration of H_2O_2 increased to 10 μM . In contrast, the growth of the three tumorigenic sublines remained significantly elevated compared to that of untreated cognate controls (Figure 23b). Exogenous H_2O_2 also increased the invasiveness of the malignant II-4 and A-5RT3 cells, while the invasiveness of normal HaCaT and benign A-5 cells was reduced as determined by a transwell migration assay (Figure 23c). The differential growth response and invasiveness was not due to H_2O_2 -induced apoptosis, as apoptosis of the cell lines was only observed at H_2O_2 concentrations >100-fold higher than those used for the proliferation and invasion assays as determined by Annexin-V/PI FACS analysis (Figure 23d). These observations indicate that H_2O_2 differentially modulates the behavior of normal and neoplastic epithelial cells.

Next, we performed a polyethylene glycol (PEG)-switch assay on H_2O_2 - and MNNG-treated keratinocytes to identify oxidatively modified proteins. As an oxidant, H_2O_2 oxidizes cysteine residues in proteins and modifies their activities. The PEG-switch assay results in the conjugation of PEG to the oxidized cysteine residue, thereby increasing the molecular weight of the oxidized protein and slowing its migration within an SDS-PAGE gel. Proteins bordering the cell membrane are particularly susceptible to modification by extracellular H_2O_2 . Phosphatase and Tensin homolog (PTEN) and Src are well-established mediators of tumorigenesis that are membrane-associated. The oxidation of PTEN forms a disulfide bridge that inactivates its tumor-suppressive catalytic activity (Kwon et al., 2004; Lee et al., 2002); Src oxidation increases its autophosphorylation and concomitant activation (Giannoni et al., 2005; Giannoni and Chiarugi, 2014). Indeed, an elevated level of oxidized PTEN

and Src in keratinocytes treated with H₂O₂ and MNNG was detected by PEG-switch assay, as was oxidized JNK and EGFR, reflecting the promiscuous nature of H₂O₂ interactions (Figure 24a-c). MNNG alone only contributed to $\leq 4\%$ of oxidized PTEN and Src, whereas H₂O₂ contributed to $\sim 20\%$ oxidized PTEN and $\sim 10\%$ oxidized Src (Figure 24a, b). PTEN and Src oxidation was further confirmed by Oxyblot (Figure 24d). Oxidized PTEN protein migrates faster under non-reducing SDS-PAGE conditions compared to its reduced counterpart (Lee et al., 2002). To detect oxidized Src, total biotin-NM-labeled oxidized proteins were first immunoprecipitated with streptavidin-agarose beads and subsequently probed using anti-Src antibodies (Figure 24d).

PTEN and Src participate in the activation of the PI3K/PKB α and MAPK/ERK signaling pathways, which play pivotal roles in tumor development and progression. Immunoblot analysis also confirmed an increase in the activation of downstream mediators of the PI3K/PKB α and MAPK/ERK pathways (Figure 24e-h). A focused qPCR array comparing H₂O₂-treated epithelia and epithelia that were cocultured with CAFs revealed similar alterations in the expression profiles of many pro-oncogenic and pro-tumor genes. Likewise, the expression of tumor suppressors and growth inhibitory mediators was reduced.

Figure 24. H₂O₂ modifies PTEN and Src signaling in keratinocytes *in vitro*. (a-c) PEG-switch assay immunoblots for oxidized PTEN, Src, JNK and EGFR proteins in keratinocytes treated with 100 nM of H₂O₂ or 10 ng/mL of MNNG. Oxidized proteins become conjugated with PEG and have a higher molecular weight than their unoxidized counterparts. Bar charts represent the percentage of protein targets that have undergone oxidative modification. (d) Immunoblot analysis of oxidatively modified PTEN and Src in keratinocyte lysates that were subjected to the indicated treatments. (e-h), Immunoblot analysis of key signaling mediators of the PI3K and ERK pathways in keratinocytes that were subjected to the indicated treatments. Bars represent normalized densitometry measurements of phosphorylated protein targets after normalization against the cognate unphosphorylated forms. (i) A focus qPCR array on

keratinocytes subjected to the indicated treatments. The data are presented as the mean $\pm$ S.D. of 3 independent experiments. n.s. not significant, * p <0.05, ** p <0.01.

In addition to epithelial cells, H_2O_2 may promote field cancerization by affecting adjacent FIBs. We first investigated whether CCM alters the behavior of FIBs. FIBs cultured in CCM exhibited increased ROS production and elevated expression of fibroblast activated protein (FAP), a biomarker of CAFs (Figure 25a,b). This effect can be attributed, at least in part, to extracellular ROS because H_2O_2 was detected in CCM (Figure 25c), and the increase in CAF marker expression was attenuated when CCM was pretreated with the antioxidant NAC (Figure 25a,b).

Figure 25. ROS in CAF-conditioned medium has a transforming effect on fibroblasts *in vitro*. (a,b) Representative FACS histogram plots and median fluorescence intensities of human fibroblasts subjected to the indicated treatments, followed by staining with DCF and PE-conjugated anti-FAP antibody and FACS analysis. (c) A calibration curve was plotted to determine the H_2O_2 concentrations

(left). Extracellular H_2O_2 concentration in the conditioned medium of FIBs and CAFs was measured using the Amplex Red H_2O_2 assay (right). The data represent the means $\pm$ S.D. obtained from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$.

Similar experiments were also performed using H_2O_2 alone. Normal FIBs were exposed to exogenous H_2O_2 for 30 min twice daily over three days and were analyzed using FACS 24 hours thereafter; these cells also displayed CAF-like characteristics, such as elevated ROS and increased FAP levels after 1 day, and α SMA expression was increased after 3 days (Figure 26a-c). Consistently, analysis of the GSH:GSSG ratio revealed that CAFs and H_2O_2 - or CCM-treated FIBs experienced oxidative stress (reflected by a low GSH:GSSG ratio) compared with non-treated FIBs. NAC increased the GSH:GSSG ratio of CCM-treated FIBs (Figure 26d). Collectively, these observations suggest that extratumoral H_2O_2 is a transformative agent in stromal field cancerization that acts independently of other CAF-derived growth factors, stimulating genetic and phenotypic changes in the epithelial cells and normal stromal fibroblasts to generate a field effect.

Figure 26. H₂O₂ has a transforming effect on fibroblasts *in vitro*. (a,b) Representative FACS histogram plots and median fluorescence intensities of human fibroblasts subjected to the indicated treatments, followed by staining with DCF and PE-conjugated anti-FAP antibody and FACS analysis. (c) Immunoblot (top) and densitometry measurements (bottom) of αSMA obtained from lysates of CAFs and FIBs subjected to the indicated treatments. β-Tubulin from the same samples was used as a loading and transfer control. (d) Measurements of GSH/GSSG ratio in CAF and FIBs subjected to the indicated treatments. A low GSH/GSSG ratio is an indicator of oxidative stress in cells. The data are presented as the mean $\pm$ S.D. of 3 independent experiments. Statistical tests were performed against untreated FIBs. n.s. not significant, * p <0.05, ** p <0.01.

4.2.3. The oxidative transformation of FIBs to CAF-like cells was attenuated by catalase *in vivo*

To determine whether extratumoral H_2O_2 generates a field effect *in vivo*, we generated an admixed tumor xenograft containing three cell types (namely, epithelial cells, CAFs and VybrantDiO-labelled FIBs) in the presence or absence of 500U/mL extracellular catalase (Figure 27a). Cancerous A-5RT3 cells with CAFs:FIBs formed large tumors, whereas non-tumorigenic HaCaT cells with CAFs:FIBs formed small palpable neoplasms (Figure 27b). Notably, tumor growth was attenuated when catalase was present in the microenvironment (Figure 27b). Consistent with the *in vitro* observations, the tumor xenografts showed the reduced activation of key mediators of the PI3K/PKB α and ERK pathways when catalase was present (Figure 28a). The number of Ki67-positive proliferating epithelial cells was also reduced in the presence of catalase compared with the saline control (Figure 28b). Depletion of microenvironmental H_2O_2 by catalase partially restored basement membrane integrity between the epithelial layer and the adjacent stroma, as shown by a clear laminin 332 staining pattern, suggesting a reduced risk of malignancy (Figure 28b).

Figure 27. H_2O_2 promotes tumor growth *in vivo*. (a) A schematic of the experimental approach using VybrantDiO-labeled fibroblasts (FIBs) and CAFs for subcutaneous tumor xenografts in NSG mice. (b) Volume measurements of tumor xenografts that were injected with or without catalase. Statistical tests were performed against cognate saline-treated xenografts Mice, n = 6 per experimental condition, 2 tumors per mouse. The data are presented as mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$.

Figure 28. Immunoblot and immunofluorescence staining for malignant markers in composite tumor xenografts. (a) Immunoblot analysis of phospho-PTEN, phospho-Src and downstream signaling mediators from tumor lysates. Bars represent normalized densitometry measurements of phosphorylated protein targets normalized against the cognate unphosphorylated forms. (b) Immunofluorescence staining for the proliferation marker Ki67 and the basal lamina protein laminin 332. Pan-cytokeratin (CK) antibody staining was used to delineate epithelial-stromal boundaries. Sections were counterstained with DAPI. Scale bar = 100 μ m. The data are presented as the mean $\pm$ S.D. * p <0.05, ** p <0.01.

To determine whether extratumoral H₂O₂ transforms normal FIBs into a CAF-like phenotype *in vivo*, we examined intracellular ROS levels and FAP expression in VybrantDiO-labelled FIBs that were isolated from tumor xenografts by FACS analysis (Figure 29a). We observed that 10% of the cells were ROS^{high}/FAP^{high}FIBs (herein referred to as transformed FIBs, or tFIBs), compared to only 3% tFIBs in the presence of catalase (Figure 29b). This suggested that extracellular H₂O₂ in the tumor microenvironment is a transforming agent that results in the acquisition of a CAF-like state.

Next, we examined whether the tFIBs had tumor-promoting capabilities compared with the untransformed FIBs (ROS^{low}/FAP^{low}). To this end, we performed an adoptive transfer of tFIBs and untransformed FIBs into new tumor xenografts. We observed rapid xenograft growth when cancerous A-5RT3 cells were co-injected with tFIBs compared with FIBs (Figure 29c). Non-tumorigenic HaCaT cells also grew at an accelerated rate when co-injected with tFIBs compared with FIBs (Figure 29c). Immunofluorescence staining further confirmed a more proliferative and aggressive epithelial cell phenotype in the presence of tFIBs (Figure 29d). Moreover, adoptively transferred tumors comprising A-5RT3 cells and tFIBs exhibited slower growth and less invasion in the presence of extracellular catalase compared with controls (Figure 30). Taken together, our *in vitro* and *in vivo* observations suggest that ROS, specifically H₂O₂, induce an oxidative CAF-like state in FIBs that results in stromal-mediated field cancerization.

Figure 29. H₂O₂ confers a CAF-like state on fibroblasts *in vivo*, with tumor-promoting consequences. (a) Flow cytometry gating of Vybrant DiO-labelled FIBs recovered from tumor xenografts with or without catalase. Isolated FIBs were analyzed for ROS and FAP expression before the adoptive transfer of ROS^{high}FAP^{high}tFIBs and untransformed ROS^{low}FAP^{low}FIBs into new tumor xenografts. (b) Representative scatterplots for tumor cells stained with CellROX and FAP-PE antibody for the analysis of ROS and FAP expression. The percentage of cells in each quadrant is indicated. Numbers on the Y-axis indicate CellROX signal intensity. (c) Volume of tumor xenografts with adoptively transferred ROS^{high}/FAP^{high}tFIBs and untransformed ROS^{low}/FAP^{low}FIBs. (d) Immunofluorescence staining for the proliferation marker Ki67 and the basal lamina

protein laminin 332. Pan-cytokeratin (CK) antibody staining was used to delineate epithelial-stromal boundaries. Sections were counterstained with DAPI. Scale bar = 100 μ m. Mice, n = 6 per experimental condition. The data are presented as the mean $\pm$ S.D. * p <0.05, ** p <0.01.

Figure 30. Catalase attenuates the tumor-promoting effects of oxidatively transformed fibroblasts. (a) Volume of tumor xenografts with adoptively transferred ROS^{high}/FAP^{high}tFIBs in the presence or absence of catalase. **(b)** Immunofluorescence staining for the proliferation marker Ki67 and the basal lamina protein laminin 332. Pan-cytokeratin (CK) antibody staining was used to delineate epithelial-stromal boundaries. Sections were counterstained with DAPI. Scale bar = 100 μ m. Mice, n = 6 per experimental condition. The data are presented as the mean $\pm$ S.D. * p <0.05, ** p <0.01.

4.2.4. Impaired TGF β signaling reduces GPX1 expression in fibroblasts to cause a defect in ROS detoxification

Next, we investigated how extratumoral H₂O₂ was able to activate FIBs to participate in field cancerization. TGF β is a potent mediator of FIB activation that signals through the canonical Smad and non-canonical TGF β -activated kinase-1 (TAK1) pathways.

We showed that exogenous H₂O₂ had similar activating effects on FIBs, such as elevated ROS levels, increased FAP and α SMA expression and reduced GSH:GSSG ratio (Figure 25 and 26). Next, we probed the effect of H₂O₂ on fibroblast TGF β

signaling. A luciferase assay measuring the TGF β response revealed that CAFs had impaired TGF β signaling, and that FIBs became desensitized to TGF β after 2 and 3 days of H₂O₂ treatment. NAC treatment was able to restore TGF β sensitivity in CCM-treated FIBs (Figure 31a). Correspondingly, Smad3 and TGF β RII mRNA expression in FIBs decreased after 2 and 3 days of H₂O₂ treatment, and TAK1 mRNA expression decreased after 3 days of H₂O₂ treatment (Figure 31b). FIBs treated with CCM showed similar impairment of TGF β signaling, which was rescued by treatment with NAC (Figure 31a, b).

Oxidative stress also triggers NF κ B activity in fibroblasts, leading to a CAF-like phenotype.(Erez et al., 2010; Martinez-Outschoorn et al., 2014a) Indeed, FIBs treated with exogenous H₂O₂ displayed a significant increase in p65-NF κ B phosphorylation after 2 and 3 days of H₂O₂ treatment (Figure 31c). siRNA silencing of p65-NF κ B expression in FIBs partially abrogated the H₂O₂-mediated suppression of Smad3, TAK1 and TGF β mRNA and protein expression (Figure 31d-f). These findings suggest that FIBs exposed to H₂O₂ initially remain responsive to TGF β and this facilitates their activation and acquisition of FAP expression at an early time point, after just 1 day of H₂O₂ treatment. Simultaneously, H₂O₂ triggers NF κ B activation that negatively regulates TGF β signaling while potentiating FIB activation. NF κ B activation is elevated after 2 days of H₂O₂ exposure, thus NF κ B maintains FAP expression (and therefore, the CAF-like state) in H₂O₂-treated FIBs at later time points when TGF β signaling becomes impaired.

Figure 31. H₂O₂-mediated impairment of TGF β signaling in FIBs elevates ROS production, in part due to increased NF κ B signaling. (a) Relative TGF β -responsive reporter (luciferase) measurements of FIBs and CAFs subjected to the indicated treatments followed by an assay to measure the TGF β response. **(b)** Relative Smad3, TAK1, TGF β RII and GPX1 mRNA levels in FIBs and CAFs after the indicated treatments. The ribosomal RNA gene Rpl27 served as the housekeeping gene. Test for significance were performed against CAF. **(c)** Immunoblot for p65-NF κ B and phospho-p65-NF κ B expression in FIBs subjected to the indicated treatments. **(d)** An immunoblot for p65-NF κ B in FIBs and p65-NF κ B-knockdown FIBs (FIB_{NF κ B}). β -tubulin from the same samples was used as loading and transfer control. Relative mRNA **(e)** and protein **(f)** levels of Smad3, TAK1 and TGF β RII in FIBs and FIB_{NF κ B} subjected to the indicated treatments. The ribosomal RNA gene Rpl27 was used a normalizing housekeeping gene. β -tubulin from the same samples was used as loading and transfer control. The data shown represent the means $\pm$ S.D. from 3 independent experiments. * p <0.05, ** p <0.01.

To determine whether FIBs with impaired TGF β signaling promote the growth of adjacent epithelia, we performed siRNA knockdown of Smad3 (F_{Smad3}), TAK1 (F_{TAK1}) and TGF β RII (F_{TGF β RII}) in FIBs and measured mitogenic factor secretion and ROS levels. The efficiency of siRNA knockdown was validated by qPCR and immunoblotting experiments (Figure 32a,b). Compared with F_{ctrl}, no differences in proliferation or apoptosis of knockdown fibroblasts were observed by FACS analysis (Figure 32c). We examined the secreted growth factor protein profile in the conditioned medium from these knockdown fibroblasts using ELISA. Out of a total of 76 distinct proteins screened, the expression levels of 7 secreted factors were elevated (Figure 33a). A comparative analysis revealed that these factors were differentially modulated by Smad3 and TAK1 in the fibroblasts (Figure 33a).

To further strengthen our observation, focused real-time PCR arrays comparing epithelia that were cocultured with CAFs and the various knockdown FIBs were performed. Notably, the expression profiles of many pro-oncogenic or tumor-associated genes in the epithelia cocultured with CAFs were also observed in the

epithelia cocultured with F_{Smad3} , F_{TAK1} , $F_{\text{TGF}\beta\text{RII}}$ or treated with H_2O_2 (Figure 33b).

Importantly, immunoblot analyses of the epithelial layers of organotypic cocultures of keratinocytes with various knockdown fibroblasts showed the activation of the PIK3/PKB and ERK pathways which are associated with elevated epithelial proliferation, as indicated by PCNA (Figure 33c).

Figure 32. Validation of Smad3, TAK1 and TGFβRII knockdown in FIBs and evaluation of proliferation and apoptosis (a) Relative Smad3, TAK1 and TGFβRII mRNA levels in siRNA knockdown FIBs. The ribosomal RNA gene Rpl27 served as

the housekeeping gene. **(b)** Immunoblot for Smad3, TAK1 and TGF β RII protein expression in control (scrambled) FIBs and siRNA knockdown FIBs. β -Tubulin from the same samples was used as a loading and transfer control. **(c)** FACs analyses of BrdU (top panel) and Annexin-PI (bottom panel) to determine proliferation and apoptosis status in knockdown FIBs.

Figure 33. Mitogenic factor array of F_{ctrl} , F_{Smad3} , F_{TAK1} and $F_{TGF\beta RII}$ conditioned media and immunoblot analyses to determine mitogenic signaling in co-cultured keratinocytes. (a) Mitogenic factor array analyses of the 48 hour conditioned medium from FIBs or knockdown FIBs. The data are presented as the mean $\pm$ S.D. of 3 independent experiments. Statistical tests were performed against F_{ctrl} . * $p < 0.05$, ** $p < 0.01$. C, Immunoblot analyses of the indicated proteins in keratinocytes from OTCs that were cocultured with FIBs or indicated knockdown FIBs. β -tubulin from the same samples was used as loading and transfer control.

Next, we measured ROS production in knockdown fibroblasts. Compared with F_{ctrl} , ROS production was elevated in F_{Smad3} (8.9-fold), F_{TAK1} (8.4-fold) and $F_{TGF\beta RII}$ (8.2-fold) (Figure 34a,b). We also detected a significant increase in extracellular H_2O_2 from the knockdown fibroblasts compared with F_{ctrl} . The concentration of extracellular H_2O_2 was determined based on a calibration curve that was created using known H_2O_2 concentrations (Figure 34c). $F_{TGF\beta RII}$ produced the highest concentration of extracellular H_2O_2 (Figure 34c).

To investigate how impaired TGF β signaling contributes to increased ROS production in FIBs, we examined the expression of key enzymes affecting H_2O_2 detoxification in FIBs, namely, catalase, superoxide dismutase (SOD) and glutathione peroxidase (Gpx), using real-time PCR. No significant changes were observed in the mRNA levels of SOD1, SOD2 or catalase (Figure 34d). Interestingly, the mRNA and protein expression levels of Gpx1 were reduced in F_{Smad3} , F_{TAK1} and $F_{TGF\beta RII}$ compared with F_{ctrl} (Figure 34d). H_2O_2 treatment of FIBs also resulted in an initial spike followed by a gradual loss of Gpx1 expression, corresponding to impaired TGF β signaling (Figure 31b). Consistent with the qPCR data, Gpx protein expression and activity were reduced in CAFs and fibroblasts with impaired TGF β signaling, suggesting that the *GPx1* gene may be regulated by the Smad3- and TAK1-mediated pathways (Figure 34e,f). These observations suggest that epithelial cells are bathed in

an oxidative microenvironment that is supplemented with proliferative growth factors conditioned by stromal fibroblasts with impaired TGF β signaling.

Figure 34. ROS elevation in FIBs with defects in TGF β signaling is due to GPx1 deficiency. (a) A FACS histogram plot of ROS in FIBs with siRNA knockdown of Smad3, TAK1 and TGF β RII, as determined by DCF staining. The values in brackets indicate the mean fluorescence signal intensities. (b) Median DCF fluorescence intensity presented as a bar graph. (c) Extracellular H₂O₂ in conditioned medium from various knockdown FIBs as measured using the Amplex Red assay. The figure insert shows the calibration curve that was used to for determine the H₂O₂ concentration. (d) Relative SOD1, SOD2, CAT and GPx1 mRNA levels in knockdown FIBs. Rpl27 served as the housekeeping gene. (e) Immunoblot analyses of the GPx1 protein from the indicated cell lysates. β -Tubulin from the same samples was used as a loading and transfer control. (f) GPx1 activity measurements for the indicated cells normalized against total protein in the cell lysates. The data are presented as the mean $\pm$ S.D. of 3 independent experiments. * p <0.05, ** p <0.01.

4.2.5. Regulation of Gpx1 by cJUN and Smad3 in CAFs from SCCs

In silico analysis of the human *GPx1* proximal promoter revealed two putative AP-1 binding sites and one SMAD3 binding site (Figure 35a) (Jonk et al., 1998; Seldeen et al., 2009). TAK1 is also a strong activator of c-Jun N-terminal kinase, which subsequently activates cJUN. To determine whether *GPx1* is a target gene of cJUN and Smad3, chromatin immunoprecipitation (ChIP) experiments for cJUN and Smad3 followed by re-ChIP for p300, a cJUN and Smad3 coactivator, were performed. No binding of phospho-cJUN was detected at AP1(-12), indicating that this binding site is non-functional (Figure 34b). Amplification of the AP1(-262) binding site occurred in the human *GPx1* promoter after ChIP for phospho-cJUN (Figure 35b) and re-ChIP for p300 in F_{ctrl} and F_{Smad3} , but not in F_{TAK1} and $F_{TGF\beta RII}$ (Figure 35c). Amplification of the Smad3(-172) binding site also occurred after ChIP for phospho-Smad3 (Figure 35b) and re-ChIP for p300 in F_{ctrl} and F_{TAK1} , but not in F_{Smad3} and $F_{TGF\beta RII}$ (Figure 35c). No binding was detected with preimmune serum, and there was no binding to a control sequence ~2 kb upstream of the transcriptional start site. These observations indicate that the expression of the human *GPx1* gene in fibroblasts is regulated by canonical and non-canonical TGF β -mediated signaling.

To underscore the clinical relevance of TGF β signaling in stromal fibroblasts, we examined the mRNA expression of Smad3, TAK1 and TGF β RII in laser-microdissected CAFs from human SCC and adjacent peri-normal fibroblasts (NFs) obtained from 197 patients. During microdissection, we collected the stroma (vimentin-positive) that was immediately associated with tumor epithelial cells rather than stromal tissue that was distal to the tumor, and we excluded lymphocytes and obvious vascular structures. We observed a lower expression of Smad3, TAK1 and TGF β RII, alone or in combination, in CAFs compared with NFs (Figure 36a).

Immunoblot analyses of these TGF β signaling mediators in CAFs and cognate NFs isolated from five resected SCC samples that were selected at random from the available patient sample pool showed corresponding changes (Figure 36b). ChIP and re-ChIP experiments were also performed using NFs and CAFs (patient #5; reduced TGF β RII expression).

Figure 35. Regulation of Gpx1 by cJUN and Smad3 in CAFs obtained from skin SCCs. (a) A schematic diagram of the human *Gpx1* regulatory region showing the positions of the two putative AP1 binding sites and a Smad3 binding site relative to the transcription start site. The sequences of the sites are shown below. (b) ChIP analyses of Smad3 and p-cJUN occupancy on the *Gpx1* promoter of FIBs and CAFs using anti-phospho-cJUN and anti-phospho-Smad3 antibodies, followed by (c) re-ChIP for anti-p300 in F_{Smad3}, F_{TAK1}, F_{TGFβRII} and F_{ctrl}. Preimmune serum (p.i.) was used as the control for ChIP. -/+ indicate vehicle and TGFβ treatment, respectively. The data are representative of n=3 independent experiments.

Figure 36. Impaired TGFβ signaling is a clinical feature of SCC CAFs. (a) Relative mRNA levels of TGFβ signaling mediators in a cohort of 197 patients. Fold changes are relative to the cognate NFs. Rpl27 served as the housekeeping gene. The

qPCR data shown represent n=3 experiments performed in triplicate. **(b)** The relative protein levels of TGF β signaling mediators in paired CAFs and NFs obtained from five human SCCs that were selected at random from the available sample pool for protein analysis. GAPDH from the same samples served as a loading and transfer control. The values represent the mean fold differences compared with the cognate NFs. **(c)** Representative ChIP results using anti-phospho-cJUN and anti-phospho-Smad3 antibodies, followed by re-ChIP **(d)** for anti-p300 using NFs and CAF#5. Preimmune serum (p.i.) was used as the control for ChIP. The data are representative of n=3 independent experiments. CAF#5 showed reduced TGF β RII expression.

Consistent with our earlier observations, the Smad3(-172) and AP1(-262) binding sites of the human *GPx1* promoter were occupied by Smad3:p300 and cJUN:p300 in NFs, whereas their occupancies were significantly reduced in CAFs (Figure 36c,d). These results collectively indicate that impaired TGF β signaling is a clinical feature of CAFs and is responsible for their loss of *GPx1* expression and activity, which in turn leads to microenvironmental oxidative stress and field cancerization.

4.3. Conclusions

In this chapter, we demonstrate that ROS are prevalent in the tumor microenvironment and are contributed by both tumor epithelia and CAFs. In particular, H₂O₂ acts as a field effect carcinogen and has transformative effects on adjacent normal epithelia via the oxidative modification of PTEN and Src, resulting in their inactivation and activation, respectively. In transformed epithelia, H₂O₂ potentiates proliferation and invasiveness, underscoring its relevance to carcinogenesis from pre-malignancy to aggressive SCC. On the other hand, normal dermal FIBs that have been exposed to H₂O₂ adopt a CAF-like state and exhibit elevated ROS levels. This oxidative phenotype is contingent on the H₂O₂-mediated impairment of TGF β signaling in FIBs, resulting in the loss of Gpx1 expression, which manifests as an

inability to detoxify H_2O_2 . Therefore, H_2O_2 triggers a feed-forward reaction in the stroma adjacent to tumors, enhancing CAF presence and reinforcing field cancerization. Impaired $\text{TGF}\beta$ signaling was also observed in CAFs analyzed from a cohort of 197 patient SCC biopsies. Our data suggest that H_2O_2 is a key player in field cancerization, facilitating the initiation and progression of cancer and fueling tumor-stroma co-evolution.

Chapter 5: Discussion and Future Directions

5.1. Nuclear receptors in CAFs of various cancers

CAFs are the dominant population of cells within the reactive stroma and interact intimately with the tumor epithelia (Gascard and Tlsty, 2016; Kalluri, 2016; Kalluri and Zeisberg, 2006). By functioning as a potent source of mitogenic factors, extracellular matrix and matrix remodeling enzymes, CAFs coordinate multiple aspects of cancer malignancy and are associated with poor prognosis in patients. The pro-tumor secretory phenotype of CAFs relies on underlying transcriptional programs that are still not fully understood. Although transcription is generally considered a non-druggable cellular event in pharmacology (Darnell, 2002; Yan and Higgins, 2013), NRs which are ligand-responsive transcription factors stand out as uniquely amenable to pharmacological modulation (Sladek, 2003). Our understanding of sex steroid-hormone NR expression in the tumor epithelia of breast and prostate cancers has resulted in some of the best anticancer therapies to date. In contrast, the expression of NRs is rarely considered in the context of the tumor stroma, particularly in CAFs. In chapter 3 of this thesis, we sought to define the NR profile of CAFs from SCC of the skin and to evaluate their non-cell autonomous impact on cancerous epithelia. At the same time, we considered whether the targeting of driver NRs in CAFs could attenuate cancer hallmarks and complement conventional chemotherapy to overcome drug resistance in cancer cells.

We showed that 18 NRs are upregulated and 3 NRs are downregulated in SCC CAFs compared with peritumoral normal FIBs. To test the functional relevance of individual CAF NRs in SCC malignancy, NRs that were upregulated in SCC CAFs were subjected to siRNA knockdown, while downregulated NRs were overexpressed (NR-KD/OE-CAFs). When NR-KD/OE-CAFs were co-cultured with malignant A-5RT3 SCC cells, the A-5RT3 transcriptome responded to genetic disruption of the

CAF NR signature. In particular, the expression of five NRs in CAFs, namely, RAR β , PPAR β , VDR, GR and AR performed non-redundant roles in CAFs that, when disrupted, resulted in the simultaneous attenuation of multiple SCC hallmarks, including invasiveness, proliferation, energy metabolism and ROS production. SCC resistance to cisplatin treatment was also reduced. These outcomes were in part due to a difference in the paracrine signaling network between A-5RT3 and CAFs whose NR expression had been modified. Using small molecule agonists or antagonists that bind to these candidate NRs in CAFs, we further defined a combination therapy of cisplatin with RAR β and AR antagonists that caused A-5RT3:CAF tumor xenograft remission in immunocompromised mice. Histological analyses also revealed that the tumors treated by the combination therapy were less proliferative and less mesenchymal in nature and contained more apoptotic cells. Collectively, our findings demonstrated the usefulness of NR profiling in SCC CAFs which guided the selection of NR-directed small molecules for durable adjunctive chemotherapy.

Other studies on CAFs obtained from various other cancers have independently validated the functional importance of CAF NRs in disease progression. In breast cancer, the ablation of RAR β expression was found to delay Wnt signaling-induced mammary gland tumor formation. MMTV promoter-driven Wnt tumors in RAR β null mice were less proliferative and more apoptotic than their equivalents in wild-type controls. This was attributed to downregulation of the mitogenic and pro-survival IGF1/Akt signaling axis. Furthermore, a reactive stroma index that was calculated from the percentage of stroma area in excised tumor sections showed less CAF presence and reduced ECM production within RAR β null mice compared with their wild-type counterparts. Intratumoral angiogenesis was similarly reduced by RAR β ablation. These results suggest that RAR β is required for CAF function and

microenvironment remodelling (Liu and Giguere, 2014). Comparing MMTV-ErbB2 $RAR\beta^{+/+}$ and MMTV-ErbB2 $RAR\beta^{-/-}$ mice, a separate study corroborated the functional importance of $RAR\beta$ for the maintenance of α SMA-positive CAF populations. Importantly, the authors of this study showed that the expression of $RAR\beta$, but not $RAR\alpha$, was a distinguishing factor between normal and tumor stroma in humans (Liu et al., 2011). These observations are consistent with our discovery that SCC CAFs also overexpress $RAR\beta$, and that the antagonism of $RAR\beta$ expression or activity can reduce tumor aggressiveness.

Interestingly, a screen for transcription factor activity in CAFs obtained from invasive ductal carcinoma of the breast compared with normal FIBs obtained from reduction mammoplasty also identified several NRs that were differentially expressed between the two cell types. RAR - and VDR -mediated transcription was elevated in CAFs relative to FIBs (Siletz et al., 2013). Another study which involved NR profiling of the stroma adjacent to ER-positive breast tumors revealed four NRs that were significantly downregulated in CAFs relative to normal FIBs, namely, $ROR\alpha$, $TR\beta$, VDR and $PPAR\gamma$. The cumulative impact of this NR signature was the reinforcement of estrogen synthesis within the tumor microenvironment, via the regulation of aromatase. This finding has profound implications for therapy because the estrogen mimic, tamoxifen, is used as a first-line treatment for ER-positive breast tumors. CAFs whose NR signature upregulates estrogen production inevitably confer drug resistance on tumor cells (Siletz et al., 2013).

It should be noted that the data on the expression and activity of VDR in breast cancer CAFs obtained in the two aforementioned studies did not agree. However, there is no reason to assume that the CAFs examined in both studies were exactly the same, particularly because the ER status of the analyzed tumors was not mentioned in the

study of CAFs that were screened for transcription factor activity. This raised the intriguing notion that the transcriptional programmes governing a malignant tumor stroma may differ even between tumors arising within the same organ. Therefore, the NR profiling of CAFs might prove useful for stratifying patients to create personalized therapies.

Much less is known about the expression and functions of PPAR β in the CAFs of various cancers; the majority of previous studies have focused on the roles of PPAR β in tumor epithelia. Nonetheless, in colorectal cancer, PPAR β null mice displayed stromal aberrations in the form of tortuous intratumoral vasculature, suggesting that PPAR β plays a role in the tumor microenvironment, although CAFs were not directly implicated (Muller et al., 2008). Similarly, histological staining for PPAR β in the stroma of clinical ovarian carcinoma samples revealed lower PPAR β expression compared with that in the stroma of peri-tumor regions (Davidson et al., 2014). Hence, our findings lend support to the view that reduced PPAR β expression in CAFs plays an important role in tumorigenesis.

Next, the expression of GR has been studied in CAFs obtained from breast and colorectal tumors. In breast cancer, GR was prominently expressed by CAFs in 100% of 19 breast tumors of the luminal A subtype. CAF GR expression also correlated with tumor grade and proliferation index in the tumors examined, but not with ER, PR or HER2 status (Catteau et al., 2016). In another study, GR expression was higher in the tumor stroma of breast tumors than in peri-normal tissue (Smith et al., 2007). Although these studies are mainly associative, they highlight a role for glucocorticoids in the modulation of the breast tumor microenvironment. Meanwhile, in the colon, prolonged inflammation is sufficient to induce carcinogenesis. Hence, the inflammation-suppressive functions of glucocorticoids intuitively suggest that GR

activation could alleviate tumor formation. Only recently have the effects of glucocorticoid activation in CAFs been studied. Human colorectal cancer CAFs treated with dexamethasone, a potent GR agonist, displayed a dose-dependent decrease in tenascin-C, HGF and TGF β expression, indicative of GR-mediated reduction of invasion (Drebert et al., 2015).

Finally, *in vitro* studies using CAFs that were explanted from prostate tumors demonstrated that the expression of AR in CAFs promotes PC3 cell colony formation, proliferation and invasion (Yu et al., 2013a). However, an *in vivo* study using composite xenografts of prostate cancer cells admixed with AR-deficient FIBs and injected subcutaneously into the dorsal flanks of immunocompromised mice showed that stromal AR deficiency protected prostate cancer epithelia against androgen deprivation-induced apoptosis. The same study highlighted that the loss of AR expression in prostate tumor CAFs correlated with higher disease mortality in patients with castration-resistant prostate cancer (Leach et al., 2015). Prostate stromal AR expression has also been shown to decrease from benign to castration-sensitive neoplasia, and is further decreased upon the transition to castration-resistant prostate cancer (Singh et al., 2014). Our work and that of others highlight the highly heterogeneous nature of CAFs, especially since their NR expression varies not only by tumor type, but also by tumor stage or grade. Clearly, these findings warrant deeper investigation into the pattern of CAF NR expression in malignancy and its impact on tumor progression.

5.2. Nuclear receptors and the cancer-wound connection

In our study, the expression of PPAR β , VDR and GR in skin SCC CAFs was reduced compared with that in normal FIBs. Thus, agonists of these CAF NRs had

limited efficacy in blocking CAF-mediated tumorigenesis. In contrast, the expression of RAR β and AR which was elevated in CAFs relative to FIBs rendered CAFs susceptible to antagonist treatment and resulted in a potent antitumor effect when used in combination with cisplatin. Notably, all five candidate NRs identified in our study play important roles in skin homeostasis, especially during wound healing (Rieger et al., 2015). Because cancers have been described as "wounds that do not heal" (Dvorak, 2015), our work strongly suggests that the deregulation of these NRs is in part responsible for the manifestation of many pathological skin conditions, including cutaneous SCC.

Retinoids are currently used for the treatment of a spectrum of skin disorders including acne, psoriasis and hyperkeratosis. The RAR β transcript is expressed at low levels in FIBs, and treatment with retinoids upregulates the expression of RAR β in FIBs but not keratinocytes (Elder et al., 1991). Although it remains unclear whether the upregulation of RAR β in FIBs represents a myoFIB or a CAF-like state, the antagonism of RAR β , which is highly expressed in SCC CAFs, could attenuate tumorigenesis. Additionally, RAR signaling crosstalks with the TGF β pathway in mesenchymal stem cells and keratinocytes (Dingwall et al., 2011), suggesting that RARs may be involved in ECM production and migration. Both processes are highly relevant to wound healing and cancer.

During wounding, the expression of PPAR β in keratinocytes is highest during the inflammation phase and is required for the positive regulation of keratinocyte survival, adhesion and migration. PPAR β suppresses PTEN and upregulates integrin-linked kinase and phosphoinositide kinase 1 expression in keratinocytes, which in turn activate PI3K/Akt signaling to produce anti-apoptotic and pro-migratory effects (Montagner and Wahli, 2013). Elevated levels of keratinocyte-derived IL-1 β further

act in a paracrine fashion on the underlying dermal FIBs which express the cognate IL-1 receptor (IL-1r). In FIBs, IL-1 β :IL-1r binding activates the TAK1 and AP-1 axis to enhance growth factor production, thus stimulating keratinocyte proliferation. As a negative feedback measure, FIBs produce the secreted IL-1 receptor antagonist, sIL-1ra, which acts as a decoy receptor to attenuate paracrine IL-1 β signals from keratinocytes. This prevents prolonged mitogenic signaling within the wound microenvironment and facilitates a transition back to normal skin homeostasis. sIL-1ra is a direct target gene of PPAR β , and the deletion of FIB PPAR β severely diminished sIL-1ra production (Chong et al., 2009). The loss of function of PPAR β in FIBs is thus detrimental to wound healing and potentially predisposes the skin to carcinogenesis.

VDR activation by its physiological ligand, 1,25-dihydroxyvitamin D₃, promotes differentiation and inhibits proliferation in keratinocytes; both processes are necessary for the normalization of re-epithelialization. VDR-mediated cell cycle arrest in keratinocytes depends on the tumor suppressor protein, retinoblastoma, and is reinforced by the activation of cyclin-dependent kinase inhibitors (Bouillon et al., 2008). Hence, the loss of VDR signaling in the epidermis is permissive to tumor formation. In skin FIBs, liganded VDR signaling crosstalks with the canonical TGF β /Smad3 signaling axis to promote granulation tissue formation and contraction to accelerate wound healing. Interestingly, impaired stromal TGF β signaling is associated with carcinogenesis (Bhowmick et al., 2004a; Cheng et al., 2005), although whether this is a consequence of VDR downregulation needs to be explored further. Nevertheless, these results suggest that VDR activity in both the epidermis and the dermis of the skin is essential for the maintenance of skin homeostasis (Luderer et al., 2013).

GR exerts predominantly anti-inflammatory effects in the skin, though its prolonged activation leads to thinning of the skin and impaired wound closure, both of which arise from epidermal and dermal atrophy (Quax et al., 2013). However, in mice expressing a mutant form of GR that was impaired in its DNA binding capacity, there was excessive formation of granulation tissue within wounds, attributed to enhanced FIB proliferation and migration, although differences in re-epithelialization were insignificant between mutant mice and wild-type controls. These GR mutant FIBs also displayed elevated TGF β and fibronectin secretion and had a highly contractile phenotype that was reminiscent of myofibroblasts and CAFs (Grose et al., 2002). These observations suggest that the loss of genomic GR activity in FIBs confers a CAF-like state, in agreement with our finding that GR is downregulated in SCC CAFs.

Although the skin is generally not considered a sex steroid hormone-sensitive organ, keratinocytes and dermal FIBs do express AR and display cell type-specific responses to testosterone. In general, AR expression is inhibitory to wound closure, and its antagonism (e.g., by castration) promotes keratinocyte migration, improves matrix deposition and remodeling, and reduces inflammation (Ribeiro et al., 2011; Rieger et al., 2015). A study involving the cell-type specific deletion of AR in keratinocytes, FIBs and macrophages determined that FIBs AR expression was specifically responsible for the suppression of re-epithelialization (King, 2010). Similarly, CAFs with elevated AR expression may be necessary for the maintenance of a wound-like status in tumors. Our findings implicate CAF NRs in the development and progression of SCC and may provide alternative explanations for the observed commonalities between cancer and impaired wound healing.

5.3. Nuclear receptor ligands as complements to cytotoxic chemotherapy

Chemotherapy is a first-line treatment for aggressive SCC. Despite the outstanding efficacy of chemotherapy in regressing primary tumors, tumor relapse often occurs, indicating tumor heterogeneity that is permissive to residual disease. Hence, even as the range of chemotherapy options for SCC increases, the rapid onset of resistance undermines disease-free survival. Cisplatin chemotherapy is one example where intrinsic or acquired resistance by tumors severely limits the drug's long-term clinical utility (Kartalou and Essigmann, 2001). Recent evidence also shows that the tumor microenvironment is indispensable for creating a protective niche that promotes cancer cell survival in response to cisplatin chemotherapy. Once again, CAFs have been implicated in conferring cisplatin chemoresistance to tumors. CAFs elevate pro-survival STAT3 and PI3K/Akt signaling in tumor epithelia, thus dampening their response to cisplatin treatment (Zhou et al., 2016). CAF-mediated induction of tumor EMT and the generation of a cancer stem cell (CSC) phenotype were also found to reduce cancer cell sensitivity to cisplatin (Shintani et al., 2016).

In our *in vitro* experiments, SCC CAFs doubled the IC₅₀ of cisplatin in A-5RT3 cells. Chemoresistance in A-5RT3 cells was abrogated by the knockdown of PPAR β / δ , RAR β and VDR in SCC CAF, while CM from CAFs that had been treated with the RAR β antagonist, LE135, and the AR antagonist, bicalutamide, could no longer induce cisplatin resistance in A-5RT3 cells. Notably, our microarray results identified a strong upregulation of HGF, ZEB1, IL6 and POSTN in A-5RT3 cells that had been co-cultured with SCC CAFs. The expression of each of these molecules is associated with the acquisition of resistant phenotypes in various cancers. HGF binding to its receptor, c-MET, triggers the PI3K/Akt and MAPK pathways which inhibit apoptosis upon chemotherapy challenge (Gao and Vande Woude, 2005). IL6

enacts multidrug resistance via similar intracellular cascades (Ghandadi and Sahebkar, 2016). ZEB1 is a driver of EMT and an inducer of the cancer stem cell (CSC) phenotype. Post-EMT cells and CSCs are chemorefractory and are notoriously difficult to destroy (Voulgari and Pintzas, 2009). Finally, POSTN is elevated in the CSC niche, promotes cancer cell survival and facilitates metastatic colonization (Hong et al., 2013). Compatible with the above findings, when RAR β , PPAR β/δ , VDR, GR or AR expression in SCC CAFs was disrupted, the expression of HGF, ZEB1, IL6 and POSTN in A-5RT3 cells was significantly reduced, indicating less chemoresistance.

In vivo, combination treatments using low-dose cisplatin with LE135 and/or bicalutamide as adjuvants (CL, CB or CBL therapy) resulted in significant A-5RT3:CAF xenograft sensitization to cisplatin compared with cisplatin alone controls (CV). Tumor relapse upon the withdrawal of treatments occurred for all combination therapies tested. However, when the treatments were resumed, CV-treated tumors continued to grow, whereas CL therapy inhibited tumor growth, and CBL therapy resulted in a near-complete remission of tumors. Importantly, the sustained accumulation of cisplatin adducts in CBL-treated tumors indicated that cisplatin resistance was reduced. This synergy between the NR ligands and cisplatin could be immensely useful in a clinical setting.

Our data illustrate that guided by NR profiling in CAFs, additive or synergistic effects can be achieved when NR ligands are used as adjuvants to cytotoxic chemotherapy. Our approach could become a viable alternative to targeting FAP activity, which is a key strategy in therapies directed at CAFs. Notably, sibrotuzumab, an FAP neutralizing antibody, failed to achieve even one complete or partial remission in a phase II trial involving 25 patients with metastatic colon cancer (Hofheinz et al., 2003). Another phase II trial involving Talabostat, a small molecule inhibitor of FAP,

also yielded disappointing patient outcomes (Narra et al., 2007). Conceivably, the pharmacological targeting of CAFs is more effective when used as an adjunct to cytotoxic chemotherapy. Complementary and combination therapies based on a deeper understanding of CAFs warrant further investigation.

5.4. Microenvironmental oxidative stress and the antioxidant effects of nuclear receptors

Oxidative stress is another feature of the tumor microenvironment that is becoming increasingly relevant to the malignant progression of cancer. Several studies have identified CAFs as producers of and responders to ROS, which contribute to the ability of CAFs to drive tumor-stroma co-evolution (Arcucci et al., 2016; Costa et al., 2014). In chapter four of this thesis, we showed that ROS are prevalent in the stroma of a variety of human cancers. CAFs enriched the tumor microenvironment with H_2O_2 which, by virtue of its diffusible nature, acts as a field effect carcinogen. H_2O_2 differentially influenced the proliferation and invasiveness of cancer cells at various stages of progression. Normal epithelia displayed limited enhancement of proliferative and invasive potential when compared with their cancerous counterparts. We further demonstrated that extratumoral H_2O_2 from either epithelial tumor cells or CAFs reinforced the already dysregulated epithelial-mesenchymal communication to promote field cancerization. The prolonged exposure of primary human keratinocytes to low levels of H_2O_2 maintained the elevated levels of oxidatively modified PTEN and Src proteins, which triggered oncogenic signaling to promote epithelial transformation and growth. The oxidization of the tumor suppressor PTEN and oncogenic Src results in their inactivation and activation, respectively.

Lending support to a pro-mitogenic role of H₂O₂, it was shown that H₂O₂ antagonized the cytostatic functions of TGFβ1 by promoting Smad3 phosphorylation through the activation of Akt-ERK1/2-linked signaling (Choi et al., 2013). Although the precise target of H₂O₂ was not identified in that study, many studies have shown that Akt-ERK1/2 are downstream mediators of Src and PTEN (Logue and Morrison, 2012; Steelman et al., 2011). Similarly, the migration of MCF-7 breast cancer cells was enhanced by elevated Nox4 expression in a human mammary FIB cell line (Tobar et al., 2010). Nox4 predominantly produces H₂O₂ rather than O²⁻ (Bedard and Krause, 2007). These effects are in addition to the known consequences of direct interaction between ROS and DNA which increases genomic instability in the tumor, resulting in the generation and selection of resistant clones.

Our study also elucidated a role for H₂O₂ in the field cancerization of the adjacent native stroma, i.e., FIBs within the skin dermis. FIBs exposed to H₂O₂ or CM obtained from CAFs acquired an oxidative CAF-like state that exacerbated field cancerization. This phenotypic change was abrogated by the pre-treatment of CAF CM with antioxidants. Importantly, FIBs with high ROS and FAP expression that were recovered from *in vivo* tumor xenografts resulted in increased malignancy when adoptively transferred into new tumor xenografts. We further showed that impaired TGFβ1 signaling is a feature of CAFs from SCCs and was responsible for the persistent leaching of extracellular H₂O₂ into the extracellular space. The underlying mechanism involved the loss of GPx1, a key enzyme in the detoxification of H₂O₂ in biological systems, due to attenuated Smad3- and TAK1-mediated signaling. Accordingly, we detected an increase in pro-mitogenic factors in the conditioned media harvested from keratinocytes that were cocultured with FIBs with impaired TGFβ signaling. The reduced expression of Smad3, TAK1 or TGFβRII was also

detected in CAFs from a cohort of 197 SCC patients, suggesting that this impaired TGF β signaling is a clinical feature of skin SCC CAFs. Collectively, these data indicated that CAFs and cancer cells engage redox molecular circuitries, in addition to paracrine mitogenic signaling, to reinforce their reciprocal relationship.

Our observation that CAFs are impaired in their TGF β signaling may seem counter-intuitive at first, because TGF β is a well-known activator of FIBs leading to their conversion into myofibroblasts. However, it should be noted that TGF β signaling operates within a negative feedback loop that is mediated by the inhibitory Smad, Smad7, (Papageorgis and Stylianopoulos, 2015; Stroschein et al., 1999) which is upregulated by TGF β via the pro-inflammatory transcription factor NF κ B (Bitzer et al., 2000; Freudlsperger et al., 2013). Oxidative stress is also strongly associated with NF κ B activation (Gloire et al., 2006; Kratsovnik et al., 2005). Hence, FIBs exposed to H₂O₂ are initially responsive to TGF β which facilitates their activation by tumor epithelia- and CAF-derived TGF β . Simultaneously, H₂O₂ triggers NF κ B activation to suppress TGF β signaling and GPx1 expression while potentiating FIB activation. In mature tumors, TGF β is abundant and the tumor microenvironment is chronically subjected to low-grade inflammation and redox imbalance. These combined characteristics synergistically create an ecosystem that encourages the conversion of FIBs to CAFs, then renders CAFs refractory to TGF β signaling and highly oxidative due to *Gpx1* deficiency. One study has also shown that TGF β sensitizes FIBs to apoptosis under conditions of H₂O₂ exposure (Yasuda et al., 2003), lending support to the view that impaired TGF β signaling in CAFs may be necessary for their survival within an oxidatively-stressed tumor microenvironment. The FIB-specific deletion of TGF β RII in mice also led to spontaneous tumors in the prostate and forestomach, which was attributed, in part, to the activation of paracrine HGF signaling (Bhowmick

et al., 2004a; Cheng et al., 2005). Such evidence point towards a tumor-suppressive role for TGF β signaling in the stroma.

Consistent with the above observations, we detected an increase in pro-mitogenic factors such as HGF and FGF7 in the conditioned media harvested from keratinocytes cocultured with fibroblasts with impaired TGF β signaling. These elevated mitogenic factors, together with extracellular H₂O₂, promoted cancer cell proliferation, as supported by the activation of pro-oncogenic PKB α , ERK and JNK pathways in the epithelia. Our findings that the oxidative action of H₂O₂ also enables CAF-mediated field cancerization suggest that anticancer strategies that singularly target the ligand-receptor pathways may not be effective. For example, a neutralizing antibody to HGF exhibited potent antitumor effect in the treatment of glioblastoma multiforme (Cao et al., 2001). However, tumor growth became HGF-independent after repeated treatments (Zhao et al., 2010). Conceivably, the promiscuous nature of H₂O₂ interactions with cellular signaling components, especially membrane-associated molecules such as EGFR and Met, renders the cells less dependent on ligand-activated pathways to promote transformation and growth. Indeed, initial efforts focused on the inhibition of PDGF with a single agent, imatinib, generated poor outcomes (Ostman and Heldin, 2007; Wen et al., 2006). In contrast, combination treatment with hydroxyurea and imatinib has provided very encouraging findings (Reardon et al., 2005). Interestingly, hydroxyurea is a potent antioxidant that has been shown to increase the expression of *Gpx1* in a p53-dependent manner, among other effects (Koncic et al., 2011). Our work suggests that the targeting of multiple redox-sensitive pathways complements individual cytokine-directed therapy in the arrest of field cancerization and progression to malignancy.

Impaired TGF β signaling in CAFs leading to GPx1 deficiency is not the only driver of oxidative stress within the tumor microenvironment. We showed that NRs in CAFs also influence the cellular redox state of A-5RT3 tumor cells in co-culture. The knockdown of RAR β in CAFs increased intracellular ROS production in A-5RT3 cells, whereas the overexpression of VDR in CAFs had the reverse effect. These observations indicate that disrupting the expression or activity of NRs in CAFs may have direct or indirect antioxidant effects on the tumor microenvironment.

Various studies on different cancers and other disease contexts have similarly highlighted the strong antioxidant functions of NRs and their cognate ligands. A study on benzo(a)pyrene-induced lung cancer found that oxidative damage caused the loss of lung alveolar architecture and resultant carcinogenesis. Retinoids stimulated the upregulation of the ROS-detoxifying enzymes catalase, SOD and GPx in mouse lungs and livers (Ramya et al., 2012) to restrict carcinogenesis. However, it was not shown whether these ROS-detoxifying enzymes were direct target genes of the RARs or RXRs, nor did the authors demonstrate that FIBs were responsible for the antioxidant response induced by retinoids. Similarly, in a separate study, pretreatment of melanoma and glioma cells with retinoids buffered against the pro-oncogenic oxidative modification of protein kinase C (Gundimeda et al., 1993).

Vitamin D and its analogs also positively regulate the expression of the antioxidant enzymes SOD1 and SOD2 in normal and cancerous prostate epithelia (Lambert et al., 2006). Moreover, in prostate, breast and colon cancer cell lines, the enzymes required for the maintenance of reduced thioredoxin and glutathione (thioredoxin reductase 1 and glucose-6-phosphate dehydrogenase, respectively) were upregulated upon treatment with calcitriol (Fleet et al., 2012). Reduced thioredoxin and glutathione act as intracellular scavengers of ROS and are essential for redox

homeostasis. Although only glucose-6-phosphate dehydrogenase is a direct target gene of VDR, it has been suggested that vitamin D and its analogs may indirectly stimulate an antioxidant response via the induction of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression. It is also worth mentioning that retinoids and vitamin D both confer UV photoprotection in the skin, at least in part due to their antioxidant properties (Dinkova-Kostova, 2008). Finally, our laboratory has previously shown that GPx1 and catalase are direct target genes of PPAR β in FIBs, and the treatment of ROS-laden diabetic wounds with GW501516, a PPAR β agonist, resulted in faster wound closure (Wang et al., 2015). This is consistent with our NR profile which revealed that PPAR β expression is low in CAFs, thus it is conceivable that in addition to impaired TGF β signaling in CAFs, the loss of PPAR β is a "second-hit" that further depletes GPx1 expression in the stroma.

A unified view of both parts of this thesis places the profiling of NR expression in CAFs at the center of both therapeutic and preventive disease intervention. For therapy, the selection of NR ligands based on NR expression in CAFs opens a new range of adjuvants that can be administered alongside cytotoxic agents such as cisplatin. This approach of complementary chemosensitization has the potential to extend the durability of existing chemotherapy regimens and reduce the risk of minimal residual disease. For the prevention of field cancerization, the targeting of NRs that directly or indirectly modulate the expression of ROS-detoxifying enzymes in CAFs could alleviate the spread of oncogenic H₂O₂ to adjacent, uninvolved epithelia and stroma. This has important implications for tumor recurrence after surgical resection or a successful initial response to first-line treatment. Therefore, the use of NR-modulating agents represents a two-pronged approach to the management

of cancer, limiting the supportive role of the tumor microenvironment while simultaneously restraining ROS-mediated field cancerisation.

5.5. Future directions

Going forward, our future studies will incorporate the effects of ROS on field cancerisation and the tumor microenvironment into the global view of metastasis. Metastasis is the leading cause of disease mortality in cancer patients and is considered untreatable due to the wide dissemination of tumor cells across multiple tissues and organs. Metastasis is a multistep process wherein hyperproliferative cancer epithelia first undergo EMT and adopt a motile state which allows them to disregard the boundaries of the basal lamina to invade and gain access to vasculature. Intravasation then occurs, during which the cancer epithelial cells successfully endure anoikis, avoid immune detection and arrive at a secondary site at which they extravasate and become embedded in a new environment. The seed-soil hypothesis by Paget et al (Paget, 1989) accessibly describes this colonization of metastatic niches, with cancer epithelial cells being analogous to seeds and the conducive microenvironment being the equivalent of fertile soil.

An alternative perspective on metastasis is the anatomical/mechanical hypothesis which suggests that the location of the metastatic site is determined by the vasculature and lymphatic drainage from the tumor (Langley and Fidler, 2011). Although the seed-soil and anatomical/mechanical hypotheses are often depicted as competing theories on the site of metastasis, they need not be mutually exclusive. For example, the lungs and liver are frequent target sites of metastasized tumors. Both organs are highly vascularized, thus aligning with the anatomical/mechanical hypothesis. However, it is interesting to note that the lungs and liver are also highly

susceptible to chronic, low-grade inflammation and oxidative stress, thereby suggesting that a predisposed field may facilitate metastatic colonization of said organs. Conceivably, the two hypotheses fall on the same spectrum of organ susceptibility to metastatic tumors.

Therefore, our aim is to further our understanding of the role of ROS in "fertilizing" the soil at primary and metastatic sites, and to detail the influence of ROS especially in the remodeling of the tumor microenvironment at metastatic sites. In doing so, we hope to identify ROS-dependent vulnerabilities that may be exploited to impede various points in the multistep process of metastasis.

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END

SCIENTIFIC REPORTS

OPEN

Angiopoietin-like 4 Mediates Colonic Inflammation by Regulating Chemokine Transcript Stability via Tristetraprolin

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Many gastrointestinal diseases exhibit a protracted and aggravated inflammatory response that can lead to hypercytokinaemia, culminating in extensive tissue damage. Recently, angiopoietin-like 4 (ANGPTL4) has been implicated in many inflammation-associated diseases. However, how ANGPTL4 regulates colonic inflammation remains unclear. Herein, we show that ANGPTL4 deficiency in mice (ANGPTL4^{-/-}) exacerbated colonic inflammation induced by dextran sulfate sodium (DSS) or stearic acid. Microbiota was similar between the two genotypes prior DSS challenge. A microarray gene expression profile of the colon from DSS-treated ANGPTL4^{-/-} mice was enriched for genes involved in leukocyte migration and infiltration, and showed a close association to inflamed ulcerative colitis (UC), whereas the profile from ANGPTL4^{+/+} littermates resembled that of non-inflamed UC biopsies. Bone marrow transplantation demonstrates the intrinsic role of colonic ANGPTL4 in regulating leukocyte infiltration during DSS-induced inflammation. Using immortalized human colon epithelial cells, we revealed that the ANGPTL4-mediated upregulation of tristetraprolin expression operates through CREB and NF- κ B transcription factors, which in turn, regulates the stability of chemokines. Together, our findings suggest that ANGPTL4 protects against acute colonic inflammation and that its absence exacerbates the severity of inflammation. Our findings emphasize the importance of ANGPTL4 as a novel target for therapy in regulating and attenuating inflammation.

An aggravated inflammatory response is a common feature of many gastrointestinal disorders, such as inflammatory bowel diseases, enteritis, and colitis. Many of these conditions are caused by changes in dietary fat intake, the ingestion of bacteria-contaminated food and water, and certain chemicals. These insults trigger an inflammatory response by inducing the recruitment of macrophages to the site of inflammation to combat pathogens, neutralize harmful immunogens and promote tissue repair¹. However, a protracted inflammatory response can cause tissue damage and lead to hypercytokinaemia, which is a potentially fatal immune reaction. Immune cell infiltration into the site of damage is highly regulated by chemotactic factors, such as macrophage inflammatory protein 1 α and chemokine (C-C motif) ligand 2 (CCL2)^{2,3}. As the initial cellular barrier that encounters luminal insults, intestinal and colonic epithelia play important roles in the early recruitment of inflammatory cells to the mucosa. Epithelial cells are a major source of chemoattractants, and epithelial chemokine production has been proposed as a key target of future therapies for gastrointestinal disorders⁴. However, much remains to be understood about the mechanisms that regulate the levels of these chemokines in the gastrointestinal and colonic tracts.

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Angiopoietin-like 4 (ANGPTL4) is a matricellular protein that has been implicated in many inflammation-associated diseases⁵. Native full-length ANGPTL4 (fANGPTL4) is proteolytically cleaved into two functionally distinct isoforms: the N-terminal domain (nANGPTL4) inhibits lipoprotein lipase (LPL) and directly regulates energy homeostasis, while the C-terminal domain (cANGPTL4) has been implicated in various processes such as cancer metastasis, skin wound and pulmonary inflammation^{6–8}. Diabetic wounds show low endogenous cANGPTL4 levels and have been associated with an elevated F4/80⁺ macrophage population at the wound site. The infiltration of F4/80⁺ macrophages was reduced upon treatment of diabetic wounds with recombinant cANGPTL4 when compared with saline⁹. ANGPTL4 can also protect against the severe pro-inflammatory effects of saturated fat by inhibiting fatty acid uptake by mesenteric lymph node macrophages¹⁰. Similarly, ANGPTL4 confers protective effects against the development of atherosclerosis¹¹, which has been associated with atherogenesis and macrophage polarization¹². ANGPTL4 has also been identified as an angiogenic mediator in arthritis¹³. ANGPTL4 has been observed to exacerbate influenza-associated inflammation through IL-6–Stat3 signaling in the lung¹⁴. Furthermore, serum ANGPTL4 was associated with the C-reactive protein level in type II diabetic patients, suggesting that ANGPTL4 may be involved in the progression of inflammation during metabolic syndrome¹⁵. Thus, ANGPTL4 may exert both anti- and pro-inflammatory effects in a context-dependent manner. Despite numerous reports of the role of ANGPTL4 in inflammation, the mechanisms whereby ANGPTL4 modulates inflammation in various diseases remain largely unclear.

Herein, we describe an anti-inflammatory role for colonic ANGPTL4 in dextran sulfate sodium salt (DSS)-induced colitis and dietary stearic acid (SA) intake *in vitro* and *in vivo*. We showed that the microbiota was similar between ANGPTL4^{+/+} and ANGPTL4^{-/-} mice at steady states, but with perturbation such as DSS treatment some differences in microbiota community become accentuated. Bone marrow transplantation and microarray analysis confirmed the intrinsic role of colonic ANGPTL4 in regulating leukocyte infiltration during DSS-induced inflammation, and thus the colonic inflammatory landscape. The underlying mechanism involves the regulation of tristetraprolin (TTP or ZFP36), an mRNA-binding protein that is involved in chemokine destabilization, by ANGPTL4 via activation of CREB and NF- κ B transcription factors.

Results

ANGPTL4 reduces DSS- and saturated fat-induced colonic inflammation. We first characterized the intestinal and colonic tract of unchallenged ANGPTL4-knockout (ANGPTL4^{-/-}) and wild-type (ANGPTL4^{+/+}) mice. There was no significant difference in body weight, colon length, disease activity index (DAI), endpoint macroscopic scores or histological scores between the genotypes (Fig. 1a and Supplementary Fig. S1a,c). Detailed examination revealed that ANGPTL4^{-/-} mice exhibited an increased muscularis thickness and shorter colonic villus length than ANGPTL4^{+/+} littermates (Fig. 1b, Supplementary Fig. S1d,e). To gain insights into the role of ANGPTL4 in acute colonic inflammation, we challenged ANGPTL4^{-/-} and ANGPTL4^{+/+} mice to either 5% DSS or 15% (w/w) stearic acid (SA) for 8 days.

ANGPTL4^{-/-} mice receiving DSS lost significantly more weight compared with ANGPTL4^{+/+} mice from day 6 onwards (Supplementary Fig. S1a). DSS-treatment resulted in a significant increase in both the DAI and endpoint macroscopic score (Fig. 1c and Supplementary Fig. S1b) as well as a reduction in the ANGPTL4^{+/+} colonic villus length, whereas there was no significant reduction observed in ANGPTL4^{-/-} mice compared with their cognate controls (Fig. 1b and Supplementary Fig. S1e). Histological analysis of the DSS-challenged ANGPTL4^{-/-} colon sections revealed a severe loss of epithelial integrity and a massive infiltration of immune cells into the lamina propria when compared with cognate ANGPTL4^{+/+} colon (Fig. 1b). Indeed, we observed a higher number of Ly6G⁺ neutrophils and CD68⁺ macrophages in the colons of DSS-treated ANGPTL4^{-/-} mice (Fig. 1d and Supplementary Fig. S1f). FACS analysis also confirmed the higher number of Ly6G⁺, CD11b⁺ and F4/80⁺ cells in ANGPTL4^{-/-} mice compared to ANGPTL4^{+/+} mice (Fig. 1e,f). Such differences were not attributed to an increase in apoptotic cells between the genotypes, as indicated by TUNEL-positive cells and the expression of cleaved caspase 3 (Supplementary Fig. S1g). Consistent with the elevated inflammation status, we also detected higher levels of TNF- α , IL-6 and IFN- γ mRNA transcripts (Fig. 1g).

As expected, stearic acid induced a more subdued inflammatory response compared to the DSS challenge (Fig. 1g). No significant differences in weight gain, blood glucose levels, DAI, endpoint macroscopic scores or histological scores were observed between ANGPTL4^{-/-} and ANGPTL4^{+/+} mice on the SA diet compared with mice fed a normal chow diet (Fig. 1c and Supplementary Fig. S1a–c,h). ANGPTL4^{+/+} mice on an SA diet exhibited a longer colon, a thicker muscularis wall and a shorter colonic villus length at the endpoint compared with mice fed a standard diet (Fig. 1a,b and Supplementary Fig. S1c,d). Immunofluorescence staining showed a similar elevated number of Ly6G⁺ neutrophils and CD68⁺ macrophages in ANGPTL4^{-/-} colon (Fig. 1d and Supplementary Fig. S1f). FACS analysis also detected an elevated number of Ly6G⁺, CD11b⁺ and F4/80⁺ cells in ANGPTL4^{-/-} mice (Fig. 1e,f). Together, our data demonstrated that the absence of ANGPTL4 enriched for a subset of the immune cell population not limited to neutrophils, macrophages and eosinophils during inflammation. Altogether, our observations are consistent with a model whereby ANGPTL4 attenuates colonic inflammation in response to inflammatory stimuli.

ANGPTL4 deficiency does not influence colonic commensal microbiota at steady state. Several reports have suggested that dysbiosis aggravates host inflammatory and metabolic diseases^{16–19}. Studies also suggested that a subset of microbes could regulate ANGPTL4 expression in the gut in response to changes in energy demands^{20–22}. To examine if the exacerbated colonic inflammatory response in ANGPTL4^{-/-} mice could be attributed to microbe composition, we performed 16S metagenomics sequencing using the V4 region to identify microbe abundance between ANGPTL4^{+/+} and ANGPTL4^{-/-} littermates before and after DSS-induced inflammation. Redundancy analysis showed that genotype and inflammation amounted to 41.2% of the total variation observed between microbial compositions in the colon. Genotype could only explain for 10.7% of the differences

Figure 1. ANGPTL4 attenuates colonic inflammation. (a–e) Images of colon samples (a) Representative colon sections stained with hematoxylin and eosin (H&E) (b). (c) Disease activity index (DAI) of mice assessed every alternate day over the treatment regimes. Scoring criteria can be found in Supplementary Table S1. (d) Percentage of Ly6G⁺ and CD68⁺ cells per field of view. Total DAPI-stained nuclei were taken as the total cell number. Microscopic views from 5 different sections were numerated. (e) Representative FACS histograms for Ly6G expression (neutrophils) from ANGPTL4^{+/+} and ANGPTL4^{-/-} littermates for the indicated treatments. (f) Representative FACS analysis of the colons from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice double stained for F4/80 (macrophages) and CD11b (macrophages, monocytes, granulocytes, NK cells, dendritic cells) for each of the treatments. (g) Relative mRNA levels of pro-inflammatory cytokines (TNF-α, IL-6 and IFN-γ) from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice at the endpoint. For the Veh, DSS and SA groups, n = 20 mice were used for each treatment. The Mann–Whitney U test was used. *p < 0.05; **p < 0.01.

Figure 2. ANGPTL4 deficiency does not influence commensal microbe composition. Pie charts showing the commensal microbe landscape at steady state (*upper panel*) and after DSS treatment (*lower panel*) of age- and gender-matched $ANGPTL4^{+/+}$ and $ANGPTL4^{-/-}$ littermates. Values in the charts denote mean percentage of abundance. $n = 6$ mice per group.

observed, while inflammation amounted to at least 31.1%. The steady state phylogenetic microbe composition was predominated by Firmicutes and Bacteroidetes, which did not differ much between both $ANGPTL4^{+/+}$ and $ANGPTL4^{-/-}$ mice before DSS treatment (Fig. 2). As expected, DSS-induced inflammation caused a sharp decrease in Bacteroidetes and a concomitant increase in Firmicutes and Proteobacteria. We found that Firmicutes were more abundant in inflamed $ANGPTL4^{+/+}$ mice, while Proteobacteria was more prevalent in $ANGPTL4^{-/-}$ mice (Fig. 2). Collectively, our data showed that microbe composition was similar between $ANGPTL4^{+/+}$ and $ANGPTL4^{-/-}$ mice at steady state, but with perturbation such as DSS treatment, some differences in the microbiota community become accentuated.

Intrinsic role of colonic ANGPTL4 in leukocytes infiltration during DSS-induced inflammation.

To further define the role of epithelial-derived ANGPTL4, we performed bone marrow transplantation (BMT) experiment. We transferred bone marrow from $ANGPTL4^{+/+}$ and $ANGPTL4^{-/-}$ donors to γ -irradiated wild-type $ANGPTL4^{+/+}$ (WT IR) recipient mice (BMT ($ANGPTL4^{+/+}$) or BMT ($ANGPTL4^{-/-}$); Fig. 3a,b). $CD4^{+}$ and $CD8^{+}$ cells were depleted from bone marrow inoculum before BMT to limit possible graft-versus-host-disease (GVHD)-related mortality. The bone marrow of WT IR mice appeared necrotic with a significant reduction in the number of bone marrow cells. The spleen was smaller in size and has reduced $CD45^{+}$ splenic cells (Fig. 3c,d). Hematopoietic reconstitution was observed through the repopulation of bone marrow cells in the femur and $CD45^{+}$ cells in the spleen at 4-week post BMT (Fig. 3c,d). Genotype PCR confirmed the successful reconstitution of the respective donor cells in WT IR mice (Fig. 3e). No significant difference in classical clinical scorings was observed between BMT ($ANGPTL4^{+/+}$) or BMT ($ANGPTL4^{-/-}$) chimeras on vehicle treatment (Fig. 3f). Histological analysis also revealed little difference between BMT ($ANGPTL4^{+/+}$) or BMT ($ANGPTL4^{-/-}$) chimeras (Fig. 3g). As expected, DSS-induced inflammation caused an erosion of the epithelial lining with increased infiltration of immune cells into the lamina propria (Fig. 3g). Notably, there was no significant difference recorded in the DAI, endpoint macroscopic score or histological score between BMT ($ANGPTL4^{+/+}$) and BMT ($ANGPTL4^{-/-}$) chimeras after DSS treatment (Fig. 3f,g and Supplementary Tables S1–3). FACS analysis also showed no significant difference in the number of $CD11b^{+}$ and $F4/80^{+}$ cells in the inflamed colons of BMT ($ANGPTL4^{+/+}$) and BMT ($ANGPTL4^{-/-}$) chimeras (Fig. 3h). Taken together, our data demonstrates the intrinsic role of epithelial-derived ANGPTL4 in regulating colonic leukocyte infiltration during DSS-induced inflammation, and thus the colonic inflammatory landscape.

ANGPTL4 deficiency alters colonic inflammatory gene expression. Our observations suggest that ANGPTL4 deficiency increased the susceptibility to inflammation induced by SA and DSS, albeit with differential severities. To strengthen our findings, we performed a comparative microarray gene expression analysis using colon tissues from $ANGPTL4^{-/-}$ and $ANGPTL4^{+/+}$ mice challenged with either DSS or SA. Using Ingenuity Pathway Analysis (IPA), we identified 250 differentially expressed genes associated with “gastrointestinal diseases”. As expected, the gene expression profiles of DSS-challenged colon samples showed a more aggravated

Figure 3. Intrinsic role of colonic ANGPTL4 in leukocytes infiltration during DSS. (a,b) Graphical illustrations depicting the experimental setup for bone marrow transplantation (BMT). CD4⁺ and CD8⁺ depleted bone marrow cells from age- and gender-matched ANGPTL4^{+/+} or ANGPTL4^{-/-} donors were transplanted into γ -irradiated ANGPTL4^{+/+} wild-type recipient (WT IR). Chimera BMT ANGPTL4^{+/+} and BMT ANGPTL4^{-/-} denote WT IR transplanted with bone marrow cells from ANGPTL4^{+/+} and ANGPTL4^{-/-} donor, respectively. Graph showed the number of CD45⁺ cells from spleen of indicated mice (Mean $\pm$ S.D.; n = 6 mice per group). (c,d) Representative

images of the femur (c) and spleen (d) from indicated mice (WT and WT IR) and chimeras (BMT ANGPTL4^{+/+} and BMT ANGPTL4^{-/-}). Inserts in (c) showed the bone marrow cell pellet before and after red blood cell lysis. Graph in (c; *left panel*) showed the number of bone marrow cells from femur of indicated mice (Mean $\pm$ S.D.; n = 6 mice per group). (e) Representative agarose gel image of genotype PCR products from CD45⁺ splenic cells from indicated mice. (f) Graphs showing the disease activity index (DAI; *left panel*), macroscopic score (*middle panel*) and histological score (*right panel*) of indicated mice treated with vehicle or 2% DSS. Individual scoring criteria can be found in Supplementary Tables S1–S3. (g) Representative hematoxylin and eosin (H&E) images of colon section from vehicle and DSS treated mice post BMT. (h) Representative FACS analysis for CD11b⁺ and F4/80⁺ cells from colons of BMT ANGPTL4^{+/+} and BMT ANGPTL4^{-/-} chimeras treated with vehicle or DSS. n = 6 per group. The Mann-Whitney U test was used. *p < 0.05; **p < 0.01 and ***p < 0.001. n.s. denotes not significant.

response when compared with the SA-challenged colon samples. Consistent with the above observations, the heat map also indicated that ANGPTL4 deficiency resulted in more severe inflammation in ANGPTL4^{-/-} mice when compared with ANGPTL4^{+/+} mice (Fig. 4a,b and Supplementary Table S4). Further studies of the 2 islands of dissimilarly regulated genes between either challenge groups or genotypes revealed that 52% of the transcripts were related to TNF- α signaling, whereas 27% and 31% were linked to the IL-10 and IL-6 signaling cascades, respectively (Fig. 4c). Gene ontology maps suggested that these genes were primarily involved in leukocyte migration and infiltration (Fig. 4d). The top diseases identified from previous studies listed in the IPA database include colitis, inflammation of the intestine, diabetes mellitus and gastrointestinal tract cancers and tumors (Fig. 4d). Next, a comparative gene expression analysis was performed between DSS-challenged ANGPTL4^{-/-} and ANGPTL4^{+/+} mice and human ulcerative colitis (UC) colonoscopy samples. The colonic mucosal samples from UC patient biopsies were classified as “inflamed” or “non-inflamed”, according to whether the samples exhibited signs of inflammation during collection. Unsupervised hierarchical clustering revealed that the gene expression profile of DSS-challenged ANGPTL4^{+/+} mice was more closely associated with the non-inflamed UC samples, while the profile of ANGPTL4^{-/-} mice was clustered more closely with the inflamed UC samples (Fig. 4e and Supplementary Table S5). Further analysis revealed that the UC samples showed the highest number of up-regulated genes, followed by the DSS-challenged ANGPTL4^{-/-} mice; the DSS-challenged ANGPTL4^{+/+} mice showed the lowest number of up-regulated genes (Fig. 4f). Our analysis also identified a cluster of distinctively regulated genes across both the mouse and human samples. Among these genes, 29%, 27%, and 23%, were involved in the IL-4, IL-1 β and IL-6 signaling cascades, respectively (Fig. 4g). Gene ontology analysis revealed that the top molecular and cellular functions included cellular movement, cellular development, cellular function and maintenance, and cellular proliferation, as well as cell death and survival (Fig. 4h). These transcripts have also been found to be involved in myeloid cell homeostasis, immune responses, and leukocyte homeostasis, activation and migration (Fig. 4h). Together, the histological analysis and gene expression profiling indicated that ANGPTL4^{-/-} mice were more susceptible to colonic inflammation, implicating a role for ANGPTL4 in immune cell infiltration.

ANGPTL4 deficiency increases the infiltration of immune cells. ANGPTL4 deficiency exacerbated colonic inflammation upon DSS and SA challenge. Guided by histological differences and gene expression changes between colon samples from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice, we next investigated the chemokine expression profile during inflammation. Consistent with the microarray findings, we detected an overall elevated expression of pro-inflammatory chemokines and a reduced expression of anti-inflammatory cytokines IL-10 and IL-17 in ANGPTL4^{-/-} mice compared with their ANGPTL4^{+/+} littermates (Fig. 5a). The highest levels of these chemokines were also consistent with greater infiltration of inflammatory cells into the colons of ANGPTL4^{-/-} mice (Fig. 1d–f and Supplementary Fig. 1f). The protein concentrations of CCL2, CCL11 and CXCL10 were also found to be elevated in ANGPTL4^{-/-} littermates in both the DSS- and SA- treated groups (Supplementary Fig. S2a). Furthermore, we observe a decreasing trend, albeit not statistically significant, in the protein levels of IL-1 β , IL-10 and IL-17 between ANGPTL4^{+/+} and ANGPTL4^{-/-} littermates among different treatments.

To date, little is known about the mechanisms by which ANGPTL4 exerts such effects on the immune and chemokine landscape. Recent publications suggest that ANGPTL4 may modulate the inflammatory response in a variety of disease models^{9–11,14,15,23}. Our microarray analysis and a review of the literature directed us to two potential intermediary proteins that respond quickly during acute inflammation and acts broadly to attenuate inflammation^{24,25}. Human antigen R (HuR) and tristetraprolin (TTP) are mRNA binding proteins well-characterized for their opposite roles in binding mRNA at AU-rich regions located at the 3′-UTR of target transcripts, including chemokines, to modify the inflammatory landscape at the onset of inflammation. HuR is highly selective in its interaction with its cognate mRNA binding partners and has been described as an active participant in promoting mRNA stability^{26,27}. In contrast, TTP promotes mRNA degradation by destabilizing target transcripts^{28–30}. The acute inflammation elicited by DSS and SA treatments significantly suppressed both ANGPTL4 and TTP expression in ANGPTL4^{+/+} littermates (Supplementary Fig. 2b). However, we detected no significant change in HuR expression in both pro-inflammatory treatments. Analysis of our focused gene expression array revealed that the expression of TTP was altered, as was the expression of genes involved in immune cell infiltration and responses. The reduced mRNA and protein levels of TTP in ANGPTL4^{+/+} and ANGPTL4^{-/-} littermates were confirmed by real-time PCR and western blot analyses (Supplementary Fig. 2c). Together, our data suggests the involvement of TTP in the regulation of local colonic inflammation.

To further understand the role that colonic epithelial cells play in regulating chemokine expression, we treated human colon epithelial cells (iCECs) with a panel of pro-inflammatory (DSS, SA, IL-1 β or TNF- α) or

Figure 4. ANGPTL4 deficiency alters colonic inflammatory gene expression. (a,e) Using Partek Genomic Suite software, we generated a microarray heat map showing changes in gene expression among colon tissues from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice fed with Veh, DSS or SA (a) and among colons from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice treated with DSS, inflamed and non-inflamed colon biopsies from ulcerative colitis patients (GSE9452) (e). Transcripts were estimated using a log₂ transformation and subjected to unbiased ANOVA to detect differentially expressed genes between samples. Only genes with a fold change < -1.2 or > 1.2 were of significance. The genes were then hierarchical clustered based on significance to generate heatmaps. For (a), among the 434 transcripts associated with “gastrointestinal diseases” identified with the IPA database,

63% were found to be commonly regulated across the three treatment groups. For (e), 32% of the transcripts associated with “gastrointestinal diseases” were found to be shared among the groups, with the gene profile from DSS-induced inflammation in $ANGPTL4^{-/-}$ mice most closely associated with the inflamed UC biopsies. (b,f) A larger number of up-regulated genes was correlated with an increasing severity of colonic inflammation in the microarray analysis. (c) Gene ontology analysis of gene clusters (black boxes in (a)) indicated that the major pathways included IL-6-, IL-10- and TNF-related pathways. (d) IPA analysis ranked colitis, inflammation of the intestine and inflammatory bowel disease as the most closely associated gastrointestinal diseases, with leukocyte migration and infiltration as the predominant functions. (g) Among the genes that were commonly regulated between human and mouse colon samples (black box), most genes encode for components of the IL-1 β , IL-6 and IL-4 pathways. (h) The IPA database ranked cellular movement, development, function, growth and proliferation as the top molecular processes involved, along with immune processes such as leukocyte activation, myeloid cell homeostasis and immune responses being the most significant biological functions involved. The numbers in the bar graphs in (d,h) represent the p-values of the process in the GO analysis, with the smallest p-value being the most significant. An in-depth overview is described in Supplementary Fig. S4.

anti-inflammatory (HC or NaBu) stimuli. Notably, $ANGPTL4$ and TTP levels decreased when iCECs were treated with DSS, SA, IL-1 β and TNF- α (Fig. 5b). Exposure to such pro-inflammatory stimuli increased the levels of CCL2, CCL11, CXCL10 and IL-1 β mRNA transcripts and decreased the levels of IL-10 and IL-17 mRNA transcripts (Supplementary Fig. 2d). Conversely, anti-inflammatory HC and NaBu increased both $ANGPTL4$ and TTP levels, prompting a decreased expression of CCL2, CCL11, CXCL10 and IL-1 β concomitant with an increase in IL-10 and IL-17 (Fig. 5b and Supplementary Fig. 2d). Next, we examined the role of $ANGPTL4$ and TTP in mediating the abundance and stability of chemokines. iCECs were subjected to transient $ANGPTL4$ (iCEC- $C_{ANGPTL4}$) or TTP knockdowns (iCEC- TTP) and subsequently exposed to pro- or anti-inflammatory stimulation (Fig. 5c and Supplementary Fig. S2e–g, Tables S6 and 7). As a control, scrambled siRNA was used (iCEC- C_{ctrl}). Pro-inflammatory stimuli (DSS, SA, IL-1 β and TNF- α) increased CCL2, CCL11, CXCL10 and IL-1 β expression but decreased IL-10 and IL-17 in iCEC- C_{ctrl} . Conversely, HC and NaBu stimulation in iCEC- C_{ctrl} suppressed CCL2, CCL11, CXCL10 and IL-1 β but heightened IL-10 and IL-17. The absence of $ANGPTL4$ (iCEC- $C_{ANGPTL4}$) raised the basal levels of CCL2, CCL11, CXCL10, IL-1 β , IL-10 and IL-17 expression independent of the nature of stimulation. Interestingly, the CCL11, IL-10 and IL-17 levels surged independent of stimulation when TTP was ablated (iCEC- TTP). Further investigation into chemokine stability demonstrated that the half-lives of CCL11, IL-10 and IL-17 mRNA transcripts, but not CCL2, IL-1 β or CXCL10, were prolonged in iCEC- $C_{ANGPTL4}$ and iCEC- TTP (Fig. 5d). The decreased half-life of TTP in iCEC- $C_{ANGPTL4}$, and not vice versa, also confirmed that $ANGPTL4$ positively regulates downstream TTP (Supplementary Fig. S2g). In an effort to understand immune cell infiltration, THP1-derived macrophages were co-cultured with iCECs (iCEC- $C_{ANGPTL4}$, iCEC- TTP and iCEC- C_{ctrl}) in the presence of various treatments, and macrophage transwell migration was investigated. Consistent with the above results, we found that deficiency in $ANGPTL4$ and TTP was sufficient to increase chemotactic signals from epithelial cells and accelerate the migration of macrophages (Fig. 5e and Supplementary Fig. S2i). Taken together, our results demonstrate that $ANGPTL4$ is an important regulator that modulates the chemokine landscape and immune cell infiltration during inflammation. Our data also highlights the role of $ANGPTL4$ in attenuating inflammation simultaneously through TTP-dependent and independent pathways, although the mechanism for the latter remains to be elucidated.

$ANGPTL4$ up-regulates TTP via CREB and NF- κ B. Full-length $ANGPTL4$ is proteolytically cleaved into N-terminal coiled-coil (n $ANGPTL4$) and C-terminal fibrinogen-like (c $ANGPTL4$) fragments³¹. To understand which fragment of $ANGPTL4$ is responsible for regulating TTP expression, we treated iCECs with recombinant (rh) c $ANGPTL4$ and rh-n $ANGPTL4$. This investigation showed that c $ANGPTL4$ was potent in significantly up-regulating TTP expression (Supplementary Fig. 3a). Next, we sought to elucidate the mechanism whereby $ANGPTL4$ regulates TTP expression. We subjected rh-c $ANGPTL4$ -stimulated iCECs to a kinase inhibitor array screen to identify key signaling pathways involved in the c $ANGPTL4$ -mediated up-regulation of TTP (Supplementary Fig. 3b). TTP expression was increased when iCECs were treated with rh-c $ANGPTL4$ (Supplementary Fig. 3b, left panel). Hence, we reasoned that kinase inhibitors that negated or inhibited the rh-c $ANGPTL4$ -mediated up-regulation of TTP mRNA transcripts indicated the involvement of those specific kinases in the selected signaling cascades (middle panel). Conversely, kinase inhibitors that did not attenuate the c $ANGPTL4$ -mediated up-regulation of TTP would indicate that those kinases were not involved in $ANGPTL4$ -mediated signaling (right panel). Our results showed that inhibitors against CDK, JNK, MEK1/2, mTOR, PI3K δ , TYK2, BTK, CK2, GSK3, PDK1 and Aurora A/B/C targeted key signaling regulators of c $ANGPTL4$ -mediated TTP transcription (Supplementary Fig. 3c). To avoid bias, all kinases that prevented the up-regulation of TTP expression under the stimulation of rh-c $ANGPTL4$ were used to study how their availability impacted the overall level of inflammatory signaling. Using IPA, we further established that these signaling mediators resulted in the activation of downstream transcription factors, such as AP1 (cFos-cJun complex), CREB and NF- κ B (Fig. 6a). Immunoblot analysis revealed that rh-c $ANGPTL4$ increased the phosphorylation of CREB (pCREB) and NF- κ B (pNF- κ B) but not phosphorylated cFos (pcFos) (Fig. 6b). *In silico* analysis identified putative CREB (+455 to +453 bp), AP1 (+388 to +395 bp) and NF- κ B (+528 to +537 bp) transcription binding sites in the promoter of TTP (Fig. 6c).

To underscore the importance of CREB and NF- κ B as active transcription factors mediating TTP expression upon c $ANGPTL4$ stimulation, we inhibited the activity of CREB, NF- κ B or both using siRNAs (iCEC- C_{RELA} , iCEC- C_{CREB} and iCEC- $C_{RELA/CREB}$) and specific kinase inhibitors (IKK-2, 666-15 and IKK-2/666-15), respectively. The highly

Figure 5. ANGPTL4 deficiency increases the infiltration of immune cells. (a) Relative mRNA levels of indicated chemokines in colon tissues from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice treated with Veh, DSS or SA. (b) Relative expression (left panel) and immunoblot analysis (right panel) of ANGPTL4 and TTP levels in iCECs when stimulated with DSS, SA, IL-1 β , TNF- α , HC or NaBu. (c) Heatmap displaying log-transformed relative expression levels of indicated chemokine mRNA transcripts in iCEC_{Ctrl}, iCEC_{TTP} and iCEC_{ANGPTL4} when stimulated with DSS, SA, IL-1 β , TNF- α , HC or NaBu. (d) Decay curves of IL-1 β , IL-10, IL-17, CCL2, CCL11 and CXCL10 in iCEC_{ANGPTL4}, iCEC_{TTP} or iCEC_{Ctrl} following actinomycin D treatment after NaBu stimulation. (e) Transwell migration assay measuring the percentage change in relative fluorescence intensity of THP1 migration in response to chemokines secreted by iCEC_{Ctrl}, iCEC_{TTP} and iCEC_{ANGPTL4} (bottom panel). Five independent experiments (n = 5) were performed in triplicate. The Mann–Whitney U test was used. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 6. ANGPTL4 up-regulates TTP expression via CREB and NF-κB. (a) IPA identified CREB, NF-κB and AP-1 as potential transcription factors that regulate the expression of TTP when stimulated with rh-cANGPTL4. IPA-assisted pathways were mapped following experimental screens using a kinase inhibitor array. (b) Immunoblot analysis revealed an increase in the phosphorylation of CREB (pCREB) and NF-κB p65 (pNF-κB) but not cFos (pcFos) after rh-cANGPTL4 stimulation. (c) A schematic illustration of the promoter of the human *TTP* gene. The putative transcription factors binding sites were determined *in silico* online with the

Jaspar database. The relative location of the ChIP primers was indicated. **(d)** Chromatin Immunoprecipitation (ChIP) and re-ChIP using pCREB or pNF- κ B/p65 and coactivator p300 antibodies in iCECs that were stimulated with rh-ANGPTL4. **(e)** Relative expression and immunoblot analysis of TTP for iCECs repressed of CREB and/or NF- κ B activity using siRNA (iCEC_{Ctrl}, iCEC_{RELA}, iCEC_{CREB} and iCEC_{RELA/CREB}) and specific kinase inhibitors (Veh, IKK-2, 666-15 and IKK-2/666-15) in the absence and presence of rh-cANGPTL4. **(f)** Densitometry analysis showing the relative expression of pCREB and pNF- κ Bp65 in nuclear and cytoplasmic fractions of iCECs as treated in **(e)**, normalized against Histone H3 and GAPDH for nuclear and cytoplasmic fractions, respectively, for Supplementary Fig. 3h. The Mann–Whitney U test was used. Three independent experiments (n = 3) were performed.

potent CREB inhibitor (666-15) hinders the interaction of KID-KIX, which is essential for CREB-dependent gene transcription³², while the IKK-2 inhibitor IV (IKK-2) selectively targets IKK-2 and retards downstream p65NF- κ B signaling. In both instances, the lack of either CREB or NF- κ B was sufficient to reduce TTP expression in iCECs even in the presence of rh-cANGPTL4 (Fig. 6e and Supplementary Fig. S3g). We also observed reduced pCREB and pNF- κ B levels in nuclear and cytoplasmic fractions of iCECs during cANGPTL4-stimulation, suggesting that the nuclear translocation of pCREB and pNF- κ B was severely compromised (Fig. 6f and Supplementary Fig. 3h). In conclusion, our data suggests that the CREB and NF- κ B transcription factors play irreplaceable roles in the ANGPTL4-mediated, TTP-dependent signaling axis.

Discussion

The epithelial cells that line the gastrointestinal tract serve as a physiological barrier that prevents the invasion of pathogens and a selective conduit of luminal signals to the host. When harmful immunogens and pathogens are detected, epithelial cells produce chemoattractants to initiate the infiltration of immune cells into the mucosa. Our findings highlight the importance of colonic epithelial cell-secreted ANGPTL4 as a prospective regulator that alters the chemokine landscape in the colon to influence downstream inflammation. We showed that ANGPTL4-deficient mice were more susceptible to acute DSS and SA exposure. 16S metagenomic sequencing indicated little difference in microbe composition between ANGPTL4^{+/+} and ANGPTL4^{-/-} mice that could account for the exacerbated inflammatory response in ANGPTL4^{-/-} mice. Bone marrow transplant study emphasized the intrinsic role of epithelial-derived ANGPTL4 in regulating the inflammatory landscape in the colon. Using human colonic epithelial cells, we further showed that ANGPTL4 regulated the expression of TTP, an mRNA destabilizing agent, via the activation of CREB and NF- κ B. The absence of ANGPTL4 or TTP prolonged the mRNA half-life of a specific subset of chemokines.

An exuberant and protracted inflammatory response contributes to the development of many gastrointestinal disorders. Infiltrating macrophages are the major cellular components of this inflammatory response. As the initial cellular barrier, the colonic epithelium plays an important role in the recruitment of inflammatory cells to the mucosa. We showed that ANGPTL4-knockout mice fed DSS or SA exhibited a greater colonic inflammatory response, which was associated with greater infiltration of immune cells when compared with wild-type mice fed similar treatments. Similarly, pro-inflammatory stimuli such as DSS, SA, IL-1 β and TNF- α suppressed the expression of ANGPTL4 and TTP in iCECs, whereas anti-inflammatory stimuli such as HC and NaBu increased its expression. In support of these findings, previous studies showed that anti-inflammatory glucocorticoid treatment boosted TTP expression in lymphocytes³³, while the synthetic glucocorticoid dexamethasone increased TTP levels in A549 cells³⁴ as well as ANGPTL4 levels in hepatocytes³⁵ and adipocytes³⁶. Although the exact pathways remain to be elucidated, dietary NaBu was also found to stimulate ANGPTL4 expression via a PPAR-independent pathway³⁷. HC has also been demonstrated to up-regulate IL-10 and IL-17 levels in natural killer cells to improve pneumonia survival rates³⁸, although no detailed mechanism was reported. Interestingly, our findings cement the importance of ANGPTL4 in regulating the general inflammatory landscape in the gastrointestinal tract. Although other ANGPTL4-dependent but TTP-independent signaling mechanisms remain to be identified, we demonstrated that ANGPTL4 modifies the availability of a subset of chemokine signals that alter the ease of immune cell infiltration through a TTP-dependent signaling circuit.

Confluent iCECs mimic an intact gastrointestinal mucosal layer and express low basal levels of ANGPTL4 and TTP. Many previous studies have utilized cancerous lines, such as HT-29, Caco-2 and HCT-116 cells, as surrogates for an *in vitro* gastrointestinal model^{39,40}. Potentially, this ANGPTL4-dependent mechanism may be impaired or altered in cancerous lines, as the expression of ANGPTL4 is known to be elevated in many types of cancer⁷. We further established that iCECs secreted ANGPTL4, which acted in an autocrine manner to induce the expression of TTP. We previously showed that secreted ANGPTL4 bound and activated integrin-mediated pathways⁴¹. Indeed, TTP is an important factor that contributes to mediating, modulating and attenuating inflammatory responses. Using the combination of an unbiased kinase inhibitor screen assay, along with ChIP and re-ChIP experiments, our current work unveiled multiple signaling conduits whereby ANGPTL4 stimulates TTP expression, resulting in CREB and NF- κ B activation. Interestingly, BTK and Aurora inhibitors were identified to significantly inhibit the ANGPTL4-dependent up-regulation of TTP, suggesting that they exert dominant roles in this signaling cascade. Lending support for a role for Aurora in NF- κ B activation, Katsha A *et al.* also reported that Aurora kinase A promoted and sustained inflammation through NF- κ B, leading to tumor formation⁴². Again, the importance of Aurora in the ANGPTL4-mediated up-regulation of TTP may be compromised when studied in cancer cell lines. Our study also emphasizes a potential role for Aurora kinases in regulating inflammation. Similarly, other studies have shown that cancer cells that express low levels of TTP are correlated with a genetic signature of low expression of CREB-related target genes⁴³. Numerous studies have also shown that TTP binds to AU-rich sequences at the 3'-UTRs of specific mRNA transcripts such as TNF- α ⁴⁴, IFN- γ ⁴⁵, IL-10 and IL-17⁴⁶. In

agreement with these previous reports, we showed that the mRNA half-lives of specific inflammatory chemokines were prolonged in the absence of ANGPTL4 and TTP.

The pathogenesis of inflammatory bowel disease in humans is complex and has a multifactorial and diverse aetiology⁴⁷. Dysbiosis has been associated with inflammation and impaired mucosal immune function in intestine of humans^{48–50}. Our 16S sequencing revealed little difference in microbe composition between two genotypes that can account for the exacerbated colonic inflammatory response in ANGPTL4^{-/-} mice. In this respect, the extent to which ANGPTL4 expression defines the colonic microbiota profiles remains to be delineated. It is also conceivable that mechanisms other than ANGPTL4-TTP-mediated chemokine mRNA stability might be involved. Our microarray analyses showed that Lrg1 and Gpx2 expression was up-regulated by 6- and 4-fold, respectively, in the colons of ANGPTL4^{-/-} mice compared with wild-type counterparts. Lrg1 is proposed to be a possible biomarker for the diagnosis of ulcerative colitis⁵¹. Both Lrg1 and Gpx2 proteins are up-regulated during the acute stages of pediatric appendicitis⁵² and colitis⁵³, respectively. These data suggest that additional ANGPTL4-dependent mechanisms may contribute to the severity of colitis in ANGPTL4^{-/-} mice. Furthermore, ANGPTL4 has been implicated in many inflammation-associated pathologies. Although not explored in the present study, the mechanism whereby ANGPTL4 may be targeted to influence cell infiltration via TTP-mediated chemokine mRNA stability could be applicable to these pathologies.

Materials and Methods

Antibodies and Reagents. Antibodies against c-Fos (#2250), phospho-c-Fos (Ser32; #5348), Elk-1 (#9182), phospho-Elk-1 (Ser383; #9186), CREB (#48H2) and phospho-CREB (Ser133; #9198) were from Cell Signaling (USA). Antibodies against Histone H3 (05-499), NF- κ B/p65 (MAB3026) and phospho-NF- κ B/p65 (Ser276; AB3375) were from Merck Millipore, USA. Antibodies against Ly-6G (#127602 and #127614), CD68 (#137002) were from Biolegend, USA. FACS antibodies (APC-/FITC-conjugated) against CD11b, CD45, F4/80 were from Miltenyi Biotec, USA. Anti-CD4- and anti-CD8a- conjugated microbeads (#130-049-201, #130-049-401) was purchased from Miltenyi Biotec, USA. Anti-TTP (sc-14030, Santa Cruz, USA), active/cleaved Caspase 3 (NB100-56113, Novus Biological, USA),

ChIP-grade antibodies against NF- κ B/p65 (ab7970) and CREB (ab31387) were from Abcam, UK and p300 (RW128) from Upstate Biotechnology, USA. IRDye[®] 680 LT goat anti-rabbit IgG, goat anti-mouse IgG and donkey anti-goat IgG were purchased from Li-Cor Biosciences, USA. Dextran sulfate sodium salt (DSS; 36 000 to 50 000M.Wt) was from Affymetrix, USA (#14489). Recombinant human cANGPTL4 (rh-cANGPTL4) and anti-ANGPTL4 antibodies were produced in-house as previously described²⁴. Recombinant human nANGPTL4 (rh-nANGPTL4; 8249-AN, RnD Systems), IL-1 β (ab9617, Abcam), TNF- α (#717904, BioLegend) and hydrocortisone (CAS 50-23-7, Calbiochem). All other chemicals were purchased from Sigma-Aldrich.

Animals. ANGPTL4^{+/-} C57BL/6J mice were acquired from the Mutant Mouse Regional Resource Center (MMRRC) and were generated by Genentech. The mice were crossed to produce ANGPTL4^{+/+} and ANGPTL4^{-/-} offspring. DNA from mouse ear clippings was isolated using the KAPA Express Extract reactions and genotyped using Mouse Genotyping HotStart in accordance with the manufacturer's recommendations (KAPA Biosystems, USA).

The PCR products were visualized on 2% agarose gels stained with SYBR Safe (ThermoFisher Scientific, USA). In the DSS treatment study, age-matched ANGPTL4^{+/+} and ANGPTL4^{-/-} males (n = 20) were treated with 5% DSS in the drinking water for 8 days; the DSS was changed every 2 days. In the SA study, mice (n = 20) were fed with a 15% SA:85% ground chow (w/w) diet for 8 days. The disease activity index (DAI) was assessed every alternate day, while macroscopic and histological scorings were performed at the experimental endpoint. The scoring criteria are available in Supplementary Tables S1–3. Protein and RNA samples were collected by scraping colonic epithelial cells. Animal experiments were approved by and carried out in accordance with the guidelines of Nanyang Technological University's Institutional Animal Care and Use Committee (NTU, IACUC, ARF SBS/NIE-A0321 and ARF SBS/NIE-A0324), Singapore.

Cell Culture. SV40-immortalized human colon epithelial cells (iCECs; T0570; ABM Canada) were cultured in Prigrow III Medium (ABM Canada) in collagen-coated flasks and maintained at 5% CO₂ at 37 °C. Confluent iCECs were stimulated with either 1 μ g/mL DSS, 500 μ M SA, 10 ng/mL IL-1 β , 10 ng/mL TNF- α , 0.4 μ g/mL HC or 2 mM NaBu for 6 h prior to harvesting. The siRNA knockdown against ANGPTL4, CREB, RELA or TTP was accomplished using Dharmacon ON-TARGETplus siRNA (Supplementary Table S8) according to the manufacturer's protocol (Thermo Scientific, USA). Inhibition of the transcription factors CREB and IKK were carried out using 0.1 μ M CREB inhibitor (666-15; #5661; Tocris Bioscience, UK) and/or 1 μ M IKK-2 inhibitor IV (IKK-2; 40-1481; Merck Millipore) respectively. iCEC_{ANGPTL4}, iCEC_{TTP} and iCEC_{Ctrl} were pre-treated with 2 mM NaBu for 2 h, followed by 10 μ g/mL actinomycin D for half-life measurements. *In vitro* experiments were carried out in triplicate.

RNA Extraction and Real-time Quantitative PCR. RNA was extracted using TRIzol (Invitrogen, USA) and was reverse transcribed using the iScript[™] Reverse Transcription Supermix according to the manufacturer's recommendations (Bio-Rad, USA). Quantitative PCR (qPCR) was conducted as previously described⁵⁴. Primer sequences are presented in Supplementary Table S9. The relative expression levels of the respective mRNAs described in this manuscript were normalized against those of the housekeeping ribosomal 18S RNA.

Microarray. RNA was transcribed into cDNA using an Applause WT-Amp ST System in accordance with the manufacturer's recommendations (NuGEN Technologies, USA). cDNA was purified using a MinElute Reaction Cleanup Kit (Qiagen, USA), fragmented and labeled using an Encore Biotin Module kit (NuGEN Technologies,

USA). Labeled cDNA molecules were hybridized using the Affymetrix hybridization master mix and injected onto GeneChip Mouse Gene 1.0 ST Array gene chips (Affymetrix, USA) for scanning. Partek Genomic Suite (Partek Inc., USA) and Ingenuity Pathway Analysis (IPA) software were used to perform analyses, an overview of which is available in Supplementary Fig. S4. The human colonic mucosal samples used for the comparative analyses in Fig. 3e–h were published by Olsen *et al.* under the GEO accession number GSE9452⁵⁵. The microarray data (accession number GSE78500) have been deposited in the GEO database.

Protein Extraction and Western Blot Analysis. Proteins were extracted in ice-cold lysis buffer (20 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 250 mM NaCl, 1% Triton-X, 0.1% SDS, 1 mM PMSF, and 200 μM sodium orthovanadate) as previously described⁴⁴. Equal amounts of proteins were resolved using SDS-PAGE gels and electro-transferred to low-fluorescence PVDF membranes (IPFL00010; Merck Millipore, USA). The cytoplasmic and nuclear fractions were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (#78833; Thermo Scientific). Briefly, all protein samples for western blot analysis performed in this manuscript were first normalized using the NanoDrop 3300 Spectrophotometer (Thermo Scientific) prior to the ensuing downstream analyses. All loading controls for immunoblot analyses were obtained from the same sample. Membranes were blocked with 0.5 $\times$ Odyssey Blocking Buffer (LI-COR Biotechnology, USA) and probed with the respective primary antibodies overnight at 4 °C in 1 $\times$ Blocking Buffer with 0.1% Tween-20. Membranes were washed thrice in TBST (50 mM Tris HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 5 min each and incubated for 1 h with the respective secondary antibodies in 1 $\times$ Blocking Buffer at room temperature. Each membrane was washed thrice in TBST and then air-dried. Images were analyzed using a CLx scanner and Image Studio V2.1 (LI-COR Biosciences, USA). All membranes were first probed for target proteins before being re-probed for GAPDH housekeeping protein. Densitometry analyses were processed using ImageJ (NIH, USA). Densitometry values are presented under each blot, with values in bold representing significance ($p < 0.05$), or in graphs (for Fig. 4e and Supplementary Fig. 3h). The LEGENDplex Multi-Analyte Flow Assay Kit was used to measure the protein concentrations of targets (CCL2, CCL11, CXCL10, IL-1 β , IL-10 and IL-17) for both mouse and humans and was customized through Biolegend, USA and performed in accordance with the manufacturer's recommendations.

Tissue Processing, H&E, Immunofluorescence and TUNEL Staining. Tissues were harvested and fixed in 4% paraformaldehyde overnight at 4 °C and subsequently were dehydrated and embedded in paraffin as previously described⁵⁶. Tissues were rehydrated and stained with hematoxylin and eosin (H&E) according to the manufacturer's protocol (Sigma–Aldrich, USA). Slides were mounted with Fluka Eukitt quick-hardening mounting medium (Sigma–Aldrich, USA). Images were obtained using a Zeiss Axiovert 200M microscope with a Zeiss FLUAR 10x/0.5 NA objective and PALMRobo V4.3 software (Carl Zeiss, Germany).

The TUNEL assay was performed in accordance with the manufacturer's protocol (Roche). For immunofluorescence analyses, tissue sections were rehydrated and incubated in 5% normal goat serum (NGS) for 1 h and then incubated with the primary antibodies in 3% NGS overnight at 4 °C. Subsequently, sections were washed and then incubated with respective secondary Alexa Fluor 594-conjugated antibodies (Invitrogen) for 1 hour at room temperature. Slides were then mounted with Hoechst 33342 dye (Life Technologies, USA). Images were obtained using a LSM 710 confocal microscope with a Zeiss EC Plan-NEOFLUAR 20x/0.5 NA objective and were analyzed using ZEN 2012 Blue Edition software (Carl Zeiss, Germany).

Tissue Dissociation and Flow Cytometry. Mouse colon tissues from various treatment groups were first homogenized into single-cell suspensions using the gentleMACS Dissociator (Miltenyi Biotec, USA) in DMEM containing 1 mg/mL collagenase-3 and 40 units/mL DNase 1. Homogenates were filtered through a 70 μm nylon cell strainer, washed and finally resuspended in PBS blocking buffer containing 3% FBS. The resultant suspension was then incubated with the respective FITC-/APC- conjugated antibodies on ice. Samples were washed twice and resuspended in PBS, and were then subjected to FACS analysis on the Accuri C6 Flow Cytometer (BD Biosciences, USA). All FACS analyses were performed for 5000 events.

Transwell Migration Assay. THP1 monocytes were differentiated in 100 ng/mL TPA complete medium for 48 h and allowed to recover for 24 h on transwell inserts. Respective siRNA knockdowns were performed on iCECs (iCEC_{ANGPTL4}, iCEC_{TTP} and iCEC_{Ctrl}). Inserts containing differentiated THP1 cells were then introduced to the transfected iCECs and exposed to various pro- and anti-inflammatory stimuli for 10 h (Fig. 3e). Inserts were then washed with PBS twice and fixed in 1% glutaraldehyde for 10 min, rinsed with PBS and stained with SYTO 60 (Thermo Fisher, USA) for 30 min. Cotton buds were used to remove all un migrated cells trapped in the upper chamber of the inserts. Inserts were rinsed again in PBS before the quantification of fluorescence using the CLx scanner and Image Studio V2.1 (LI-COR Biosciences, USA). Relative fluorescence as a readout for cellular migration was calculated by normalizing the intensities between test wells (THP1 and iCECs) and control wells (THP1 without iCECs).

Chromatin Immunoprecipitation (ChIP) and Re-ChIP. ChIP and re-ChIP experiments were performed as described previously⁵⁷. In addition, samples were subjected to washes with increased stringencies: twice each with the low (1% Triton X-100, 0.1% SDS, 2 nM EDTA, 20 mM Tris-HCl, 150 mM NaCl at pH 8.1) and high salt (1% Triton X-100, 0.1% SDS, 2 nM EDTA, 20 mM Tris-HCl, 500 mM NaCl at pH 8.1) wash buffers. Primer sequences are presented in Supplementary Table S9.

Kinase Inhibitor Array. Immortalized human colon cells were subjected to treatment with 95 kinase inhibitors (SYN-2103; SYNkinase, Australia) in the presence or absence of rh-c-ANGPTL4 (8 $\mu\text{g/mL}$) for 6 h (Supplementary Table S10). RNA was then isolated and reverse transcribed as described above.

16S Metagenomics Sequencing. Post-weaned, age- and gender-matched ANGPTL4^{-/-} or ANGPTL4^{+/+} mice were used. Fresh fecal pellets were collected before and after DSS treatment. Bacterial genomic DNA was isolated from feces using the FASTDNA spin kit (#116570200, MP Biomedicals, USA). 16S sequencing of the 16S V4 variable region was performed by SeqMatic LLC, USA using the Illumina MiSeq sequencing platform.

Bone Marrow Transplant Model. BMT was performed using bone marrow cells isolated from donor mice (ANGPTL4^{-/-} or ANGPTL4^{+/+}) and transplanted into recipient γ -irradiated ANGPTL4^{+/+} wild-type mice (WT IR; n = 6 per group). Recipient WT mice were kept on acidified water (pH 3.0) over the course of the BMT. WT mice were medicated with sulfamethoxazole (40 mg/kg) and trimethoprim (8 mg/kg) in oral suspension for one week prior to and after γ -irradiation (9.5 Gy) using the Biobeam 8000 (Gamma-Service Medical GmbH, Germany). Red blood cells from donor bone marrow cells were lysed (0.89% NH₄Cl, 0.1 mM EDTA, pH 7.2). Residual bone marrow cells were washed with PBS and filtered through 30 μ M nylon cell strainer before depleting CD4⁺ and CD8⁺ cell populations using the QuadroMACS kit (#130-091-051, Miltenyi Biotec, USA). Approximately 10⁷ CD4⁺ and CD8⁺ depleted bone marrow cells were introduced to WT IR mice via retro-orbital injection. Chimera mice [BMT (ANGPTL4^{+/+}) and BMT (ANGPTL4^{-/-})] were allowed to recover for 4-weeks before 2% DSS treatment for 8 days. Bone marrow reconstitution was determined using genotype PCR (Supplementary Table S11). PCR products were visualized on 2% agarose gels tinted with SYBR Safe (ThermoFisher Scientific, USA). Cell count was numerated using the automated ADAM-MC cell counter (NanoEntek, USA). Cells from the spleen and colon were analyzed for immune cell infiltration using FACS (BD LSRFortessa X-20).

Statistical Analysis. Statistical analyses were performed using two-tailed Mann-Whitney U tests. P-values < 0.05 denote statistically significant differences between means; *p < 0.05; **p < 0.01 and ***p < 0.001. Values are expressed as the means $\pm$ standard error.

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Author Contributions

N.S.T. and T.P. designed, organized and interpreted the data, and wrote this manuscript. T.P., M.K.S., E.H.P.T., D.S.L.C., Y.L., J.W.K.W., J.S.K.C. and M.M.K.L. performed the experiments. Z.T., C.K.T., P.C.Z. and V.A. assisted in data analyses. N.S.T. supervised the entire study.

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Cancer-associated fibroblasts enact field cancerization by promoting extratumoral oxidative stress

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Histological inspection of visually normal tissue adjacent to neoplastic lesions often reveals multiple foci of cellular abnormalities. This suggests the presence of a regional carcinogenic signal that spreads oncogenic transformation and field cancerization. We observed an abundance of mutagenic reactive oxygen species in the stroma of cryosectioned patient tumor biopsies, indicative of extratumoral oxidative stress. Diffusible hydrogen peroxide (H_2O_2) was elevated in the conditioned medium of cultured skin epithelia at various stages of oncogenic transformation, and H_2O_2 production increased with greater tumor-forming and metastatic capacity of the studied cell lines. Explanted cancer-associated fibroblasts (CAFs) also had higher levels of H_2O_2 secretion compared with normal fibroblasts (FIBs). These results suggest that extracellular H_2O_2 acts as a field effect carcinogen. Indeed, H_2O_2 -treated keratinocytes displayed decreased phosphatase and tensin homolog (PTEN) and increased Src activities because of oxidative modification. Furthermore, treating FIBs with CAF-conditioned medium or exogenous H_2O_2 resulted in the acquisition of an oxidative, CAF-like state. *In vivo*, the proliferative potential and invasiveness of composite tumor xenografts comprising cancerous or non-tumor-forming epithelia with CAFs and FIBs could be attenuated by the presence of catalase. Importantly, we showed that oxidatively transformed FIBs isolated from composite tumor xenografts retained their ability to promote tumor growth and aggressiveness when adoptively transferred into new xenografts. Higher H_2O_2 production by CAFs was contingent on impaired TGF β signaling leading to the suppression of the antioxidant enzyme glutathione peroxidase 1 (GPX1). Finally, we detected a reduction in Smad3, TAK1 and TGF β RII expression in a cohort of 197 clinical squamous cell carcinoma (SCC) CAFs, suggesting that impaired stromal TGF β signaling may be a clinical feature of SCC. Our study indicated that CAFs and cancer cells engage redox signaling circuitries and mitogenic signaling to reinforce their reciprocal relationship, suggesting that future anticancer approaches should simultaneously target ligand receptor and redox-mediated pathways.

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Tumor initiation, growth and propagation are frequently accompanied by desmoplasia and the acquisition of a reactive stroma mainly comprising cancer-associated fibroblasts (CAFs).^{1–3} The dynamic and reciprocal relationship between the epithelial and mesenchymal compartments of the tumor dictates almost every aspect of cancer progression, even governing the efficacy of therapy and influencing the risk of disease relapse.^{4,5} Importantly, a population of epithelial and stromal cells adjacent to the primary neoplastic lesion acquires early genetic changes but lacks histopathology, indicating that tumors prime the surrounding tissue for malignancy, an effect known as field cancerization.^{6,7} Field cancerization results in multifocal primary tumors in close proximity with a higher chance of recurrence after surgical resection of malignant tumors.⁸ Although genetic and epigenetic anomalies have been well described as the driving force behind field cancerization of premalignant epithelia,⁹ mediators of field cancerization by the stroma remain poorly understood.

The expansion of the cancer field requires soluble factors that promote oncogenic transformation in a non-cell-autonomous manner and prime the adjacent naïve stroma to support tumor growth. Although these functions are usually ascribed to growth factors and cytokines, reactive oxygen species (ROS), which are persistently elevated in almost all cancers, have been recently identified as critical intermediates of cellular signaling.^{9–11} The oxidative modification of proteins by ROS modulates intracellular signaling to influence cellular behavior and contribute to the pathophysiology of many human diseases.^{12,13} ROS are by-products of aberrant metabolism in the tumor epithelia, and their cell-autonomous mutagenic effects have been studied extensively. Much less is known about the regulation of ROS in CAFs and how ROS impact field cancerization. Notably, not all ROS are well suited for paracrine cell signaling. Superoxide anions (O_2^-) and hydroxyl radicals ($OH^\bullet$) have a very short diffusion distance because they are highly reactive, resulting in a very short half-life in the aqueous phase.¹⁴ They also have a very low permeability coefficient for lipid bilayers.¹⁵ In contrast,

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hydrogen peroxide (H_2O_2) has a longer half-life, is lipid soluble and can diffuse across the cellular plasma membrane, making it an ideal field effect carcinogen. In this study, we investigated the regulation of ROS, and in particular, H_2O_2 production, in CAFs and how this tumor stroma-mediated oxidative stress causes a premalignant field defect.

Results

Elevated ROS in the microenvironment of numerous tumor types. We first examined the oxidative status of tumor sections from patients with late stage (stage 3 to metastatic) skin squamous cell carcinoma (SCC), gastric, breast and colon cancers using the ROS-sensitive fluorescent dye CM- H_2DCFDA . Pan-cytokeratin staining distinguished between tumor epithelia and stromal elements. All tumor sections stained positive for ROS (Figure 1a). SCC sections exhibited the highest intensity of ROS staining. Interestingly, ROS were not restricted to the tumor epithelia but were frequently observed in the stromal compartments of tumors. To identify the cell types responsible for elevated microenvironmental ROS, we measured extracellular H_2O_2 in conditioned medium from various SCC lines of different aggressiveness

(Figure 1b). HaCaT is a non-tumorigenic human keratinocyte cell line, whereas benign A-5, malignant II-4 and highly aggressive A-5RT3 are sublines of different tumorigenic potential derived from HaCaT cells.¹⁶ HaCaT cells secreted the lowest amount of H_2O_2 , whereas conditioned medium from A-5RT3 cells had the highest level of H_2O_2 (Figure 1b). As CAFs dominate the tumor stroma and diffusible H_2O_2 is well suited for a role in tumor stroma crosstalk, we measured the amount of H_2O_2 in conditioned medium from CAFs explanted from four patient skin SCC biopsies alongside their paired normal fibroblasts (FIBs) (Figure 1c). We consistently detected higher extracellular H_2O_2 in the conditioned medium from SCC CAFs compared with FIBs.

Microenvironment oxidative stress causes a premalignant field defect. Next, we determined whether microenvironmental oxidative stress promotes premalignant development, that is, field cancerization. As the tumor promotion protocol involves a mutagen, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), we first determined the cytotoxic dose of MNNG in combination with various concentrations of H_2O_2 . There were no significant differences in the percentages of apoptotic (Annexin V^{high}/PI^{low} and Annexin V^{high}/PI^{high}) and

Figure 1 Elevated ROS in the microenvironment of numerous tumor types. (a) Dichlorofluorescein diacetate (DCF) staining to detect ROS on sections of patient tumor biopsies from the skin, gastric, breast and colon. Pan-cytokeratin (CK) antibody staining was used to delineate epithelial-stromal boundaries. Sections were counterstained with DAPI. Scale bar: 100 μm . (b and c) Extracellular concentrations of H_2O_2 in the conditioned medium from b, various human SCC lines, and (c) paired FIBs and CAFs from human SCC explants, as measured by Amplex Red H_2O_2 assay. * $P < 0.05$, ** $P < 0.01$

Figure 2 H_2O_2 has a transforming effect on epithelia and fibroblasts *in vitro*. (a) Quantification of epithelial colonies in soft agar at 1 month after the indicated treatments with MNNG and H_2O_2 . (b) Percentages of proliferating (S-phase) cells after the indicated treatments with H_2O_2 as determined by BrdU labeling and FACS analysis. (c) Relative fluorescence (SYTO60 dye) readings of invaded cells after the indicated treatments in the transwell invasion assay. (d) PEG-switch assay followed by immunoblot analysis of oxidatively modified PTEN and Src in keratinocyte lysates subjected to the indicated treatments. Bars represent the percentage of protein targets that have undergone oxidative modification. (e–g) Immunoblot analysis of key signaling mediators of the PI3K and ERK pathways in keratinocytes subjected to the indicated treatments. Bars represent normalized densitometry measurements of phosphorylated protein targets normalized against the cognate unphosphorylated forms. (h–k) Median fluorescence intensities of human fibroblasts subjected to the indicated treatments, followed by staining with DCF and PE-conjugated anti-FAP antibody and FACS analysis. (l) Measurements of GSH/GSSG ratio in CAF and FIBs subjected to the indicated treatments. A low GSH/GSSG ratio is an indicator of oxidative stress in cells. The data are presented as the mean $\pm$ S.D. of three independent experiments. Statistical tests were performed against cognate untreated cells for each cell type. NS, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

necrotic (AnnexinV^{low}/PI^{high}) keratinocytes treated with 1–100 nM H₂O₂ alone or in combination with 10 ng/ml MNNG (Supplementary Tables S2 and S3). Thus, this range of H₂O₂ and MNNG was used to examine the effects of H₂O₂ on

colony formation in soft agar. A marked increase in colony number was observed with cells pretreated with MNNG and exposed to H₂O₂ for 4 weeks (Figure 2a). Keratinocytes treated with a higher concentration of H₂O₂ alone formed

colonies in soft agar, suggesting that chronic exposure to elevated extracellular H_2O_2 facilitates the transformation of epithelial cells. It is conceivable that normal and neoplastic epithelial cells respond differently to H_2O_2 . Indeed, we observed differential growth response and invasiveness to exogenous H_2O_2 . The proliferation of HaCaT and A-5RT3 cells peaked at 10 nM H_2O_2 , whereas the proliferation of A-5 and IL-4 cells peaked at 100 nM H_2O_2 . The proliferation of HaCaT cells began to decrease as the concentration of H_2O_2 increased to 10 μ M. In contrast, the growth of the three tumorigenic sublines remained significantly elevated compared with untreated cognate controls (Figure 2b). Exogenous H_2O_2 also increased the invasiveness of the malignant IL-4 and A-5RT3 cells, whereas that of normal HaCaT and benign A-5 cells was reduced as determined by a transwell invasion assay (Figure 2c). The differential growth response and invasiveness was not due to H_2O_2 -induced apoptosis, as apoptosis of the cell lines was only observed at H_2O_2 concentrations >100-fold higher than those used for the proliferation and invasion assays as determined by Annexin V/PI FACS analysis. (Supplementary Figure S1A). These observations indicate that sub-cytotoxic levels of H_2O_2 differentially modulate the behavior of normal and neoplastic epithelial cells.

Next, we performed a polyethylene glycol (PEG)-switch assay on H_2O_2 - and MNNG-treated keratinocytes to identify oxidatively modified proteins. As an oxidant, H_2O_2 oxidizes cysteine residues in proteins and modifies their activities. The PEG-switch assay results in the conjugation of PEG to the oxidized cysteine residue, thereby increasing the molecular weight of the oxidized protein and slowing its migration within an SDS-PAGE gel. Proteins bordering the cell membrane are particularly susceptible to modification by extracellular H_2O_2 . Phosphatase and tensin homolog (PTEN) and Src are well-established mediators of tumorigenesis that are membrane associated. The oxidation of PTEN forms a disulfide bridge that inactivates its tumor-suppressive catalytic activity.^{17,18} Src oxidation increases its autophosphorylation and concomitant activation.^{19,20} Indeed, an elevated level of oxidized PTEN and Src in keratinocytes treated with H_2O_2 and MNNG was detected by PEG-switch assay, further confirmed by Oxyblot (Figure 2d, Supplementary Figures S1B and C). MNNG alone only contributed to $\leq 4\%$ of oxidized PTEN and Src, whereas H_2O_2 contributed to $\sim 20\%$ oxidized PTEN and $\sim 10\%$ oxidized Src (Figure 2d). Notably, oxidized JNK and EGFR were also detected by PEG-switch, reflecting the promiscuous nature of H_2O_2 interactions (Supplementary Figure S1D). PTEN and Src participate in the activation of the

PI3K/PKB α and MAPK/ERK signaling pathways, which have pivotal roles in tumor development and progression. Immunoblot analysis confirmed increased activation of downstream mediators of the PI3K/PKB α and MAPK/ERK pathways (Figures 2e-g, Supplementary Figure S1E). A focused qPCR array comparing H_2O_2 -treated epithelia and those cocultured with CAFs revealed similar altered expression profiles of many pro-oncogenic and pro-tumor genes. Similarly, the expression of tumor suppressors and growth inhibitory mediators was reduced (Supplementary Figure S1F).

In addition to epithelial cells, H_2O_2 may promote field cancerization by affecting adjacent FIBs. We first investigated whether CAF-conditioned medium (CCM) alters the behavior of FIBs. FIBs cultured in CCM had increased ROS levels and elevated expression of fibroblast-activated protein (FAP), a biomarker of CAFs (Figures 2h-i, Supplementary Figures S2A and B). This effect can be attributed, partially, to extracellular ROS because H_2O_2 was detected in CCM (Supplementary Figure S2C), and the increase in CAF marker expression was attenuated when CCM was pretreated with the antioxidant NAC. Similar experiments were also performed using H_2O_2 alone. Normal FIBs exposed to exogenous H_2O_2 also displayed elevated ROS and increased FAP levels after 1 day, and α SMA expression was increased after 3 days (Figures 2j-k, Supplementary Figure S2D-F). Consistently, analysis of the GSH:GSSG ratio revealed that CAFs and H_2O_2 - or CCM-treated FIBs experienced oxidative stress (reflected by a low GSH:GSSG ratio) compared with non-treated FIBs. NAC increased the GSH:GSSG ratio of CCM-treated FIBs (Figure 2l). These collective observations suggest that extratumoral H_2O_2 is a transformative agent in stromal field cancerization, stimulating genetic and phenotypic changes in the epithelial cells and normal stromal fibroblasts to generate a field effect.

Oxidative transformation of FIBs to CAF-like cells was attenuated by catalase *in vivo*. To determine whether extratumoral H_2O_2 generates a field effect *in vivo*, we generated an admixed tumor xenograft containing three different cell types, namely, epithelial cells, CAFs and VybrantDiO-labeled FIBs, in the presence or absence of 500 U/ml extracellular catalase (Figure 3a). Cancerous A-5RT3 cells with CAFs:FIBs formed large tumors, whereas non-tumorigenic HaCaT cells with CAFs:FIBs formed small palpable neoplasms (Figure 3b). Notably, tumor growth was attenuated by catalase in the microenvironment (Figure 3b). Consistent with the *in vitro* observations, the tumor xenografts showed reduced activation of key mediators of the

Figure 3 H_2O_2 promotes field cancerization *in vivo*. (a) Schematic of the experimental approach using VybrantDiO-labeled fibroblasts (FIBs) and CAFs for subcutaneous tumor xenografts in NSG mice. (b) Volume measurements of tumor xenografts injected with or without catalase. Statistical tests were performed against cognate saline-treated xenografts. (c) Immunoblot analysis of phospho-PTEN, phospho-Src and downstream signaling mediators from tumor lysates. Bars represent normalized densitometry measurements of phosphorylated protein targets normalized against the cognate unphosphorylated forms. (d and g) Immunofluorescence staining for the proliferation marker Ki67 and the basal lamina protein laminin 332. Pan-cytokeratin (CK) antibody staining was used to delineate epithelial-stromal boundaries. Sections were counterstained with DAPI. Scale bar = 100 μ m. (e) Representative scatterplot for tumor cells stained with CellROX and PE-conjugated FAP antibody. Only VybrantDiO-labeled FIBs were gated for analysis of ROS and FAP expression. The percentage of cells in each quadrant is indicated. The numbers in brackets on the Y axis indicate CellROX signal intensity. (f) Volume of tumor xenografts with adoptively transferred ROS^{high}/FAP^{high} FIBs and untransformed ROS^{low}/FAP^{low} FIBs. Mice, $n = 6$ per experimental condition. The data are presented as the mean $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$

PI3K/PKB α and ERK pathways when catalase was present (Figure 3c, Supplementary Figure S3A). The number of Ki67-positive proliferating epithelial cells was also reduced in the presence of catalase compared with the saline control (Figure 3d). Depletion of microenvironmental H₂O₂ by catalase partially restored basement membrane integrity between the epithelial layer and the adjacent stroma, as shown by a clear laminin 332 staining pattern, suggesting a reduced risk of malignancy (Figure 3d). To determine whether extratumoral H₂O₂ transforms normal FIBs into a CAF-like phenotype *in vivo*, we examined intracellular ROS levels and FAP expression in VybrantDiO-labeled FIBs isolated from tumor xenografts by FACS analysis (Supplementary Figure S3B). We observed that 10% of the cells were ROS^{high}/FAP^{high}FIBs, herein referred to as transformed FIBs (tFIBs), compared with only 3% tFIBs in the presence of catalase (Figure 3e). Next, we asked whether the tFIBs had tumor-promoting capabilities compared with untransformed FIBs (ROS^{low}/FAP^{low}). To this end, we performed an adoptive transfer of tFIBs and untransformed FIBs into new tumor xenografts (Figures 3f and g). We observed rapid xenograft growth when cancerous A-5RT3 cells were co-injected with tFIBs compared with FIBs. Non-tumorigenic HaCaT cells also grew at an accelerated rate when co-injected with tFIBs compared with FIBs (Figure 3f). Immunofluorescence staining further confirmed a more proliferative and aggressive epithelial cell phenotype in the presence of tFIBs (Figure 3g). Moreover, adoptively transferred tumors comprising A-5RT3 cells and tFIBs exhibited slower growth and less invasion in the presence of extracellular catalase compared with controls (Supplementary Figure S3C). Taken together, our *in vitro* and *in vivo* observations suggest that ROS, specifically H₂O₂, induce an oxidative CAF-like state in FIBs for stromal-mediated field cancerization.

Impaired TGF β signaling reduces GPX1 expression in fibroblasts to cause a defect in ROS detoxification. Next, we investigated how extratumoral H₂O₂ activated FIBs to participate in field cancerization. TGF β is a potent mediator of FIB activation, which signals through the canonical Smad and non-canonical TGF β -activated kinase-1 (TAK1) pathways. We showed that exogenous H₂O₂ had similar activating effects on FIBs, such as elevated ROS levels, increased FAP and α SMA expression and reduced GSH:GSSG ratio (Figures 2j-l, Supplementary Figure S2F). Next, we probed the effect of H₂O₂ on fibroblast TGF β signaling. A luciferase assay for TGF β response revealed that CAFs have impaired TGF β signaling, and FIBs became desensitized to TGF β after 2 and 3 days of H₂O₂ treatment (Figure 4a). Correspondingly, Smad3 and TGF β RII mRNA expression in FIBs decreased after 2 and 3 days of H₂O₂ treatment, and TAK1 mRNA expression decreased after 3 days of H₂O₂ treatment (Figure 4b). FIBs treated with CCM showed similar impairment of TGF β signaling, which was rescued by treatment with NAC (Figures 4a and b).

Oxidative stress also triggers NF κ B activity in fibroblasts, leading to a CAF-like phenotype.^{21,22} Indeed, FIBs treated with exogenous H₂O₂ displayed a significant increase in p65-NF κ B phosphorylation after 2 and 3 days of H₂O₂ treatment (Supplementary Figure S3D). siRNA silencing of

p65-NF κ B expression in FIBs partially abrogated the H₂O₂-mediated suppression of Smad3, TAK1 and TGF β mRNA and protein expression (Supplementary Figures S3E-G). These findings suggest that FIBs exposed to H₂O₂ initially remain responsive to TGF β and this facilitates their activation and acquisition of FAP expression at an early time point, after just 1 day of H₂O₂ treatment. Simultaneously, H₂O₂ triggers NF κ B activation that negatively regulates TGF β signaling while potentiating FIB activation. NF κ B activation is elevated after 2 days of H₂O₂ exposure, thus NF κ B maintains FAP expression (and therefore, the CAF-like state) in H₂O₂-treated FIBs at later time points when TGF β signaling becomes impaired.

To determine whether FIBs with impaired TGF β signaling promote the growth of adjacent epithelia, we performed siRNA knockdown of Smad3 (F_{Smad3}), TAK1 (F_{TAK1}) and TGF β RII (F_{TGF β RII}) in FIBs and measured mitogenic factor secretion and ROS levels. The efficiency of siRNA knockdown was validated by qPCR and immunoblotting (Figures 4c and d). Compared with F_{ctrl}, no differences in proliferation or apoptosis of knockdown fibroblasts were observed by FACS analysis (Supplementary Figure S4A). We examined the secreted growth factor protein profile in CCM from these knockdown fibroblasts by ELISA. Out of a total of 76 distinct proteins screened, the expression levels of 7 secreted factors were elevated (Supplementary Figure S4B). A comparative analysis revealed that these factors were differentially modulated by Smad3 and TAK1 in FIBs (Supplementary Figure S4B). To further strengthen our observation, focused real-time PCR arrays comparing epithelia cocultured with CAFs and the various knockdown FIBs were performed. Notably, the expression profiles of many pro-oncogenic or tumor-associated genes in the epithelia cocultured with CAFs were also observed in the epithelia cocultured with F_{Smad3}, F_{TAK1}, F_{TGF β RII} or treated with H₂O₂ (Supplementary Figure S1F). Importantly, immunoblot analysis of the epithelial layers of organotypic cocultures of keratinocytes with various knockdown fibroblasts showed the activation of the PI3K/PKB and ERK pathways associated with elevated epithelial proliferation, as indicated by PCNA (Supplementary Figure S4C). Next, we measured ROS levels in knockdown fibroblasts. Compared with F_{ctrl}, ROS levels were elevated in F_{Smad3} (8.9-fold), F_{TAK1} (8.4-fold) and F_{TGF β RII} (8.2-fold) (Figures 4e and f). We also detected a significant increase in extracellular H₂O₂ from the knockdown fibroblasts compared with F_{ctrl}. The concentration of extracellular H₂O₂ was derived using a calibration curve created using known H₂O₂ concentrations (Figure 4g). F_{TGF β RII} exhibited the highest amount of extracellular H₂O₂ (Figure 4g).

To investigate how impaired TGF β signaling increases H₂O₂ levels in FIBs, we examined the expression of key enzymes affecting H₂O₂ detoxification in FIBs, namely, catalase, superoxide dismutase (SOD) and glutathione peroxidase (Gpx), using real-time PCR. No significant changes were observed in the mRNA levels of SOD1, SOD2 or catalase (Figure 4h). Interestingly, the mRNA and protein expression levels of Gpx1 were reduced in F_{Smad3}, F_{TAK1} and F_{TGF β RII} compared with F_{ctrl}. H₂O₂ treatment of FIBs also resulted in an initial spike followed by a gradual loss of Gpx1 expression, corresponding to impaired TGF β signaling (Figure 4b). Consistent with the qPCR data, Gpx protein expression and activity were reduced in CAFs

and fibroblasts with impaired TGF β signaling, suggesting that the *GPx1* gene is regulated by Smad3- and TAK1-mediated pathways (Figures 4h and i). These observations indicate that

epithelial cells are bathed in an oxidative microenvironment supplemented with mitogenic factors secreted by stromal fibroblasts with impaired TGF β signaling.

Figure 4 Impaired TGF β signaling reduces GPX1 expression in FIBs to cause a defect in ROS detoxification. (a) Relative TGF β -responsive reporter (luciferase) readings of FIBs and CAFs subjected to the indicated treatments followed by assay for TGF β response. (b and c) Relative Smad3, TAK1, TGF β RII and GPX1 mRNA levels in FIBs and CAFs after the indicated treatments. The ribosomal RNA gene Rpl27 served as the housekeeping gene. (d) Immunoblot for Smad3, TAK1 and TGF β RII protein expression in control (scrambled) FIBs and siRNA knockdown FIBs. β -Tubulin from the same samples was used as a loading and transfer control. (e and f) Levels of ROS in FIBs with siRNA knockdown of Smad3, TAK1 and TGF β RII as determined by DCF staining and FACS. (e) The values in brackets indicate the mean fluorescence signal intensities. (f) Median DCF fluorescence intensity. (g) Extracellular H $_2$ O $_2$ in conditioned medium from various knockdown FIBs as measured by Amplex Red assay. The figure insert shows the calibration curve for determining H $_2$ O $_2$ concentration. (h) Relative SOD1, SOD2, CAT and GPX1 mRNA levels in knockdown FIBs. Rpl27 served as the housekeeping gene. Immunoblot of the GPX1 protein from the indicated cell lysates. β -Tubulin from the same samples was used as a loading and transfer control. (i) GPX1 activity measurements for the indicated cells normalized against total protein in the cell lysates. The data are presented as the mean $\pm$ S.D. of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001

Regulation of Gpx1 by cJUN and Smad3 in CAFs from SCCs. *In silico* analysis of the human *GPx1* proximal promoter revealed two putative AP1-binding sites and one SMAD3-binding site (Figure 5a).^{23,24} TAK1 is also a strong activator of cJun N-terminal kinase, which consequently activates cJUN. To determine whether *GPx1* is a target gene

of cJUN and Smad3, chromatin immunoprecipitation (ChIP) experiments for cJUN and Smad3 followed by re-ChIP for p300, a cJUN and Smad3 coactivator, were performed. No binding of phospho-cJUN was detected at AP1(–12), indicating that this binding site is non-functional (Supplementary Figure S5A). There was amplification of the

AP1(–262)-binding site in the human *GPx1* promoter after ChIP for phospho-cJUN (Supplementary Figure S5A) and re-ChIP for p300 in F_{ctrl} and F_{Smad3} , but not in F_{TAK1} and $F_{TGF\beta RII}$ (Figure 5a). There was also amplification of the Smad3 (–172)-binding site after ChIP for phospho-Smad3 (Supplementary Figure S5A) and re-ChIP for p300 in F_{ctrl} and F_{TAK1} , but not in F_{Smad3} and $F_{TGF\beta RII}$ (Figure 5a). No binding was detected with preimmune serum, and there was no binding to a control sequence ~2-kb upstream of the transcriptional start site. These observations indicate that the expression of the human *GPx1* gene in fibroblasts is regulated by canonical and non-canonical TGF β -mediated signaling.

To underscore the clinical relevance of TGF β signaling in stromal fibroblasts, we examined the mRNA expression of Smad3, TAK1 and TGF β RII in laser-microdissected CAFs from human SCC and adjacent peri-normal fibroblasts (NFs) from 197 patients. During microdissection, we collected the stroma (vimentin-positive) immediately associated with tumor epithelial cells rather than stromal tissue distal to the tumor, and excluded lymphocytes and obvious vascular structures. We observed reduced expression of Smad3, TAK1 and TGF β RII, alone or in combination, in CAFs compared with NFs (Figure 5b). Immunoblot analysis of these TGF β signaling mediators in CAFs and cognate NFs isolated from five resected SCC samples selected at random from the available patient sample pool showed corresponding changes (Figure 5c). ChIP and re-ChIP experiments were also performed using NFs and CAFs (patient #5; reduced TGF β RII expression). Consistent with our earlier observations, the Smad3(–172) and AP1(–262)-binding sites of the human *GPx1* promoter were occupied by Smad3:p300 and cJUN:p300 in NFs, whereas their occupancies were significantly reduced in CAFs (Figure 5d, Supplementary Figure S4B). These results collectively indicate that impaired TGF β signaling is a clinical feature of CAFs and is responsible for their loss of *GPx1* expression and activity, which consequently leads to micro-environmental oxidative stress and field cancerization.

Discussion

CAFs are one of the most abundant stromal cells in the tumor microenvironment, facilitating the development, propagation, and invasiveness of tumors.²⁵ Our study showed that CAF-derived ROS, specifically H₂O₂, induced oxidative stress in normal FIBs and trigger *de novo* CAF transformation *in vitro* and *in vivo*. Adoptive transfer of oxidatively tFIBs into malignant and non-tumorigenic epithelia xenografts resulted

in faster-growing and more aggressive tumors, indicating exacerbated oncogenic transformation. This self-amplifying, field cancerization effect was due to impaired TGF β signaling, leading to deficient *GPx1* expression and activity in CAFs. We showed that the human *GPx1* gene was regulated by TGF β 1-mediated Smad3 and TAK1/cJUN pathways in fibroblasts. Reduced expression of Smad3, TAK1 or TGF β RII was also detected in CAFs from a cohort of 197 SCC patients.

Fibroblast-specific deletion of TGF β RII in mice led to spontaneous tumors in the prostate and forestomach, which was partially attributed to the activation of paracrine HGF signaling.^{26,27} However, TGF β is required for fibroblast activation and transition to a CAF-like state. To reconcile these paradoxical observations, we noted that TGF β signaling operates in a negative feedback loop mediated by the inhibitory Smad, Smad7,^{28,29} which is upregulated by TGF β via the pro-inflammatory transcription factor NF κ B.^{30,31} Oxidative stress is also strongly associated with NF κ B activation.^{32,33} Hence, FIBs exposed to H₂O₂ are initially responsive to TGF β , which facilitates their activation by tumor epithelia- and CAF-derived TGF β . Simultaneously, H₂O₂ triggers NF κ B activation to suppress TGF β signaling and *GPx1* expression while potentiating FIB activation. In mature tumors, TGF β is abundant and the tumor microenvironment is chronically subjected to low-grade inflammation and redox imbalance. These combined characteristics synergistically create an ecosystem that encourages the conversion of FIBs to CAFs, then renders CAFs refractory to TGF β signaling and highly oxidative because of *Gpx1* deficiency. The expression of *Gpx1* is also regulated by p53,³⁴ and the ablation or mutation of p53 in stromal fibroblasts increased growth and metastatic spread of PC3 prostate cancer cells because of increased production of SDF-1.³⁵ Although the redox status of p53-deficient fibroblasts is unknown, it is conceivable that elevated H₂O₂ may contribute to their pro-tumorigenic effect. Together with our own observation that silencing NF κ B expression could prevent the H₂O₂-mediated impairment of TGF β signaling in fibroblasts, these findings suggest that transcriptional control has a critical role in modulating the pro-tumorigenic effects of CAFs.

Recent studies have revealed an important relationship between the extracellular redox state and cancer aggressiveness.⁹ The activation of oncogenes in epithelial cells results in the loss of caveolin-1 and the elevated expression of lactate transporters in stromal fibroblasts. These CAF-like fibroblasts exhibited increased ROS levels and elevated glucose uptake, which complement cancer cell metabolism.^{26,36} We showed that extratumoral H₂O₂, either

Figure 5 Regulation of *Gpx1* by cJUN and Smad3 in CAFs from skin SCCs. (a) Schematic of the human *Gpx1* regulatory region showing the positions of the two putative AP1-binding sites and a Smad3-binding site relative to the transcription start site. The sequences of the sites are shown below. Representative ChIP results using anti-phospho-cJUN and anti-phospho-Smad3 antibodies followed by re-ChIP for anti-p300 in F_{Smad3} , F_{TAK1} , $F_{TGF\beta RII}$ and F_{ctrl} . Preimmune serum (p.i.) was used as the control for ChIP. –/+ Indicate vehicle and TGF β treatment, respectively. The data are representative of $n = 3$ independent experiments. (b) Relative mRNA levels of TGF β signaling mediators in a cohort of 197 patients. Fold changes are relative to the cognate NFs. Rpl27 served as the housekeeping gene. The qPCR data shown are from $n = 3$ experiments performed in triplicate. (c) The relative protein levels of TGF β signaling mediators in paired CAFs and NFs from five human SCCs selected at random from the available sample pool for protein analysis. The immunoblot data are from three independent experiments performed in duplicate. GAPDH from the same samples served as a loading and transfer control. The values represent the mean fold differences compared with the cognate NFs. (d) Representative ChIP results using anti-phospho-cJUN and anti-phospho-Smad3 antibodies, followed by re-ChIP for anti-p300 using NFs and CAF#5. Pi. was used as the control for ChIP. The data are representative of $n = 3$ independent experiments. CAF#5 showed reduced TGF β RII expression

from epithelial tumor cells or CAFs, reinforced the already dysregulated epithelial–mesenchymal communication to promote field cancerization. Indeed, the prolonged exposure of primary human keratinocytes to low levels of H_2O_2 elevated the levels of oxidatively modified PTEN and Src, which triggered oncogenic signaling to promote epithelial transformation and growth; oxidization of the tumor suppressor PTEN and the oncogene Src results in protein inactivation and activation, respectively. H_2O_2 can also differentially influence the proliferation and invasiveness of cancer cells at various stages of progression. Lending support to a pro-mitogenic role of H_2O_2 , H_2O_2 antagonized the cytostatic functions of TGF β 1 by the activation of Akt-ERK1/2-linked signaling.³⁷ Although the precise target of H_2O_2 was not identified, many studies have shown that Akt-ERK1/2 are downstream mediators of Src and PTEN.^{34,38} Similarly, the migration of MCF-7 breast cancer cells was enhanced by elevated Nox4 expression in a human mammary fibroblast cell line.³⁶ Nox4 is known to predominantly produce H_2O_2 rather than O_2^- .³⁹

Finally, CAFs exert their proneoplastic activity by secreting mitogenic factors. Many of these factors are potential druggable targets, and it has been proposed that the inhibition of these ligand/receptor-activated signaling networks could limit neoplastic growth. We detected an increase in pro-mitogenic factors such as HGF and FGF7 in the conditioned media harvested from keratinocytes cocultured with fibroblasts with impaired TGF β signaling. These elevated mitogenic factors, together with extracellular H_2O_2 , promoted cancer cell proliferation, as supported by the activation of pro-oncogenic PKB α , ERK and JNK pathways in the epithelia. Our findings that the oxidative action of H_2O_2 also enables CAF-mediated field cancerization suggest that anticancer strategies that singularly target the ligand-receptor pathways may not be effective. For example, a neutralizing antibody to HGF exhibited potent antitumor effect in the treatment of glioblastoma multiforme.⁴⁰ However, tumor growth became HGF-independent after repeated treatments.⁴¹ Conceivably, the promiscuous nature of H_2O_2 interactions with cellular signaling components, especially membrane-associated molecules such as EGFR and Met, renders the cells less dependent on ligand-activated pathways to promote transformation and growth. Indeed, initial efforts focused on the inhibition of PDGF with a single agent, imatinib, generated poor outcomes.^{42,43} In contrast, combination treatment with hydroxyurea and imatinib has provided very encouraging findings.^{44,45} Interestingly, hydroxyurea is a potent antioxidant that has been shown to increase the expression of Gpx1 in a p53-dependent manner, among other effects.⁴⁶ Future exploration of new multi-modal approaches that simultaneously target ligand-receptor signaling and redox-mediated pathways will offer new promising anticancer strategies.

Materials and Methods

Human tumor samples. Human SCC biopsies along with their paired peritumoral tissues were provided by the National Skin Centre, Singapore. CAFs and FIBs were isolated by laser capture microdissection and subjected to protein and RNA extraction for immunoblotting and real-time PCR analyses, respectively.^{47,48} The study was approved by the National Healthcare Group Domain-Specific Review Boards (NHG-DSRB). All tumor samples were de-identified before the analyses.

In vitro tumor colony promotion protocol. Keratinocytes were seeded at a density of 1×10^5 per 35-mm dish containing complete OTC medium (Denova Sciences, Singapore) at day 0. On day 1, cultures were treated for 1 h with the initiating agent, MNNG, or solvent control (DMSO). The cells were washed, and the medium was replaced with complete medium for 24 h before exposure to different concentrations of H_2O_2 . After 1 week, cells were harvested from individual dishes and passaged at a density of 1×10^5 per 35-mm dish in H_2O_2 -containing medium. Concurrently, 2×10^4 harvested cells were embedded in 0.35% noble agar (Sigma Aldrich, St. Louis, MO, USA) and layered onto 0.7% noble agar-coated 35-mm dishes during each passage of the cells. These soft agar cultures were consistently replenished with fresh H_2O_2 -containing medium every other day. After 1 month, viable transformed colonies were stained with 1 mg/ml thiazolyl blue tetrazolium in PBS and counted.

Cell proliferation, apoptosis assays and FACS analysis. Bromodeoxyuridine (BrdU) incorporation and total DNA content in proliferating cells were analyzed by BrdU and PI staining using the BrdU Flow kit (BD Biosciences, San Jose, CA, USA). Epithelial cells were treated for 24 h with the indicated concentrations of H_2O_2 in serum-free medium. The cells were incubated with BrdU (40 μ M) for 30 min. Apoptotic and necrotic cells were quantified using an Annexin V/PI staining kit (BioLegend, San Diego, CA, USA) and subsequent FACS analysis.

PEG-switch assay and OxyBlot protein oxidation detection. PEG-switch assay was performed as previously described.⁴⁹ Cells treated with 100 nM H_2O_2 or 10 ng/ml MNNG for 24 h were lysed using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA). Oxidized thiols were reduced using DTT and alkylated using 2 mM PEG-maleimide with 0.5% SDS for 2 h at room temperature. The alkylation reaction was quenched using 100 mM Tris-HCl buffer pH 6.8, 4% SDS, 20% glycerol, and 0.01% bromophenol blue and 100 mM maleimide and the samples were resolved by SDS-PAGE and subjected to immunoblot. Oxidatively modified PTEN was analyzed as previously described with some modifications.¹⁷ Cells treated with 100 nM H_2O_2 for 30 min were washed with oxygen-free ice-cold PBS containing 1 mM N-ethylmaleimide (NEM). Cells were lysed in lysis buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 30 mM NEM). Lysates were further clarified using aQIAshredder (Qiagen, Valencia, CA, USA). Equal amounts of proteins were mixed with NEM loading dye (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 20 mM NEM and 0.02% bromophenol blue) and resolved by non-reducing SDS-PAGE. As a control, proteins were completely reduced by heating in SDS-loading dye containing DTT. Oxidized Src was detected as previously described.¹⁹ The separated proteins were subjected to immunoblot analysis using antibodies against PTEN and Src.

H_2O_2 and CCM treatment of FIBs in vitro. FIBs and CAFs were seeded at a density of 1×10^4 per 35-mm dish in FibroGRO-LS (Millipore, Billerica, MA, USA). The 48-h CCM was collected from CAFs, sterile filtered and used immediately. CCM, with or without 100 μ M N-acetylcysteine (Sigma Aldrich) pretreatment, was added to wells containing FIBs and refreshed daily over 3 days, after which FIBs were harvested for FACS analysis. Separately, FIBs were treated with 100 nM H_2O_2 in serum-free FibroGRO-LS for 30 min twice daily with an 8-h interval between treatments for a maximum of 3 days. FIBs were harvested for FACS analysis 24 h after 1 day, 2 days and 3 days of H_2O_2 treatment.

In vivo tumor growth and adoptive transfer of tFIBs. A total of 1×10^5 HaCaT or A-5RT3 cells were admixed with 2×10^5 CAFs and 1×10^6 VybrantDiO (Life Technologies, Waltham, MA, USA)-labeled FIBs in growth factor-reduced Matrigel (Corning, Tewksbury, MA, USA), with or without 500 U/ml catalase (Sigma), and implanted subcutaneously into NSG mice (Jackson Laboratory, Bar Harbor, ME, USA) (Figure 3a). Three weeks post-implantation, tumors were excised and dissociated using the MACS tumor dissociation kit and a gentleMACS Octo Dissociator (Miltenyi Biotec, Germany). Then, the tumors were doubly stained with CellROX Deep Red (Thermo, Waltham, MA, USA) and PE-conjugated anti-FAP antibody (Novus Biologicals, Littleton, CO, USA) for FACS. A total of 3×10^5 ROS^{high}/FAP^{high} tFIBs were isolated and adoptively transferred into new tumor xenografts containing either HaCaT or A-5RT3 cells. Tumor growth was monitored for 3 weeks. All animal studies were approved by the Institutional Animal Care and Use Committee of Nanyang Technological University (ARF-SBS/NIE-A0216AZ).

ChIP and re-ChIP. ChIP was performed using phospho-cJUN and phospho-Smad3 antibodies (Cell Signaling, Danvers, MA, USA), and re-ChIP was performed using a p300 antibody (Upstate Biotechnology, Lake Placid, NY, USA) as previously described.⁵⁰ The primer sequences are listed in Supplementary Table S1.

Immunoblot assay. Far-infrared immunoblotting was performed as previously described.⁵¹

Measurement of extracellular H₂O₂, intracellular ROS and GSH/GSSG ratio. Extracellular H₂O₂ and intracellular ROS were measured as previously described.^{47,52} The specificity of the assay for H₂O₂ was verified with catalase, and the degradation of H₂O₂ or inhibition of the assay system by the sample was analyzed by determining the recovery of exogenously added H₂O₂. GSH/GSSG ratio was measured using the GSH/GSSG ratio detection assay kit green (Abcam, Cambridge, UK) according to the manufacturer's recommendations.

Mitogenic factor array. The expression profiles of the secreted factors present in the conditioned medium (48 h) were identified using the RayBio Human Growth Factor Array 1 and Human Inflammation Antibody Array 3 (RayBiotech, Inc., Norcross, GA, USA). These membranes were incubated with conditioned media from the different cocultures and then processed.

Statistical analysis. Statistical differences were determined using a two-tailed Mann–Whitney U-test. A *P*-value of <0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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Conditional knockout of N-WASP in mouse fibroblast caused keratinocyte hyper proliferation and enhanced wound closure

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Neural-Wiskott Aldrich Syndrome Protein (N-WASP) is expressed ubiquitously, regulates actin polymerization and is essential during mouse development. We have previously shown that N-WASP is critical for cell-ECM adhesion in fibroblasts. To characterize the role of N-WASP in fibroblast for skin development, we generated a conditional knockout mouse model in which fibroblast N-WASP was ablated using the Cre recombinase driven by Fibroblast Specific Protein promoter (Fsp-Cre). N-WASP^{FKO} (*N-WASP^{fl/fl}; Fsp-cre*) were born following Mendelian genetics, survived without any visible abnormalities for more than 1 year and were sexually reproductive, suggesting that expression of N-WASP in fibroblast is not critical for survival under laboratory conditions. Histological sections of N-WASP^{FKO} mice skin (13 weeks old) showed thicker epidermis with higher percentage of cells staining for proliferation marker (PCNA), suggesting that N-WASP deficient fibroblasts promote keratinocyte proliferation. N-WASP^{FKO} mice skin had elevated collagen content, elevated expression of FGF7 (keratinocyte growth factor) and TGF β signaling proteins. Wound healing was faster in N-WASP^{FKO} mice compared to control mice and N-WASP deficient fibroblasts were found to have enhanced collagen gel contraction properties. These results suggest that N-WASP deficiency in fibroblasts improves wound healing by growth factor-mediated enhancement of keratinocyte proliferation and increased wound contraction in mice.

The skin is the largest organ in the body and is composed of two primary layers; the epidermis and the dermis which are separated by a basement membrane¹. The epidermis is composed mainly of keratinocytes whereas the dermis is composed mainly of connective tissue produced by dermal fibroblasts. As a protective organ, the skin requires mechanical toughness and the ability to repair itself after wounding. Wound healing can be broken down to 3 stages, the inflammatory phase, proliferation phase and the maturation phase. During the inflammatory phase, the activated platelets play an important role in the formation of a clot to stop bleeding and maintain tissue homeostasis. Both the keratinocyte and fibroblasts play a critical role during the proliferation phase. The keratinocytes proliferate and migrate to re-epithelize the epidermis layer and cover the wound while underlying fibroblasts migrate and is activated to synthesize extracellular matrix proteins for connective tissue^{2,3}. Upon tissue injury, fibroblasts differentiate into myofibroblasts and promote wound healing by decreasing the size of the wound in mice. Myofibroblasts express highly contractile protein α -smooth muscle actin (α -SMA) which provides adhesive and biochemical force required for wound closure^{4,5}. Several studies have shown the importance of fibroblasts in wound healing. Rac1 knockout in dermal fibroblast led to delay in wound healing, reduced myofibroblast activation and lowered collagen production⁶. Fibroblast specific deletion of TGF β receptor diminished the wound contraction, dermal regeneration and re-epithelialization⁷. Similarly, reduced TGF β signaling was identified in fibroblast specific integrin β 1 deficient mice⁸. All these studies suggest that the dermal fibroblast is critical for *in-vivo* tissue repair.

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Keratinocytes and fibroblasts communicate through the secretion of growth factors and cytokine⁹. During wound healing, fibroblasts within the wound bed secrete keratinocyte growth factor (KGF) that triggers keratinocytes to proliferate, facilitating re-epithelialization¹⁰. Moreover, exogenous KGF-1 expression into the excisional wounds of genetic diabetic mouse models, improved their wound healing rate and epithelialization¹¹. 12-O-tetradecanoylphorbol-13-acetate (TPA) induced epithelial cells proliferation and local inflammation was found to be reduced in the absence of proliferating fibroblast¹². In short, fibroblasts play an important role in the secretion of growth factors required for keratinocyte proliferation during the skin wound healing process.

Cell migration, adhesion, morphology, growth factor secretion, proliferation and other critical cellular processes are regulated by the actin cytoskeleton¹³. N-WASP (Neural-Wiskott Aldrich Syndrome protein) which is expressed ubiquitously and has been shown to be a critical regulator of actin cytoskeleton remodeling via Arp2/3 complex¹⁴. N-WASP deficiency in fibroblast leads to reduced cell-ECM adhesion and increased cell motility¹⁵. N-WASP regulates invadopodia formation¹⁶ in Src transformed fibroblasts^{17,18} and affects clathrin dependent¹⁹ or independent endocytosis²⁰. Moreover, N-WASP has been found to play critical role in RNA polymerase-II dependent transcription regulation, RNA processing and DNA repair via interaction with PSF-NonO complex²¹. N-WASP expression has been found to be lower in breast cancer cells²², whereas it highly expressed in hepatocellular carcinoma²³.

N-WASP deficient mice had abnormalities in neural tube formation, heart and mesoderm differentiation and die at embryonic stage E11.5^{24,25}. Conditional knockout of N-WASP expression in mouse brain leads to severe hydrocephalus condition and premature post-natal death²⁶. N-WASP is also critical for T-cells development²⁷. Conditional ablation of N-WASP expression in keratinocytes leads to ulceration, alopecia and marked epidermal hyperproliferation. This disrupted hair follicle cycling was due to increased TGF β signaling²⁸ or due to decreased Wnt-dependent transcription²⁹.

In this study we have characterized the *in-vivo* role of N-WASP in dermal fibroblasts using Fsp-Cre (fibroblast specific protein) mice to selectively ablate the expression of N-WASP in fibroblasts. N-WASP^{fl/fl}; Fsp-cre (N-WASP^{FKO}) mice were born following Mendelian genetics suggesting that deficiency of N-WASP in fibroblast does not lead to lethality. Both the male and female N-WASP^{FKO} mice were sexually active and did not show any physical abnormalities. Hematoxylin and eosin staining of skin sections of the N-WASP^{FKO} mice showed thicker epidermis suggesting that N-WASP deficiency in dermal fibroblasts leads to epidermal hyperproliferation in aged mice. Moreover, the dermal wound closure was found to be faster in N-WASP^{FKO} mice compared with the controls. *In-vitro* collagen gel contraction assay showed that N-WASP deficient fibroblast caused faster collagen gel contraction in the presence of TGF β . Expression of growth factor KGF/FGF7 was elevated in N-WASP^{FKO} mice. In addition the expression of TGF β signaling pathway proteins (Smad2, Smad3, Tak1 and TGF β -RII) was increased in N-WASP^{FKO} mice compared to control. Thus, our results suggest that the ablation of N-WASP expression in dermal fibroblasts enhanced wound healing, in part, by promoting proliferation of keratinocytes and enhanced wound contraction. Our data provide new insights into the functions of N-WASP in dermal fibroblasts regulating epidermal cell proliferation.

Results

Loss of N-WASP expression in dermal fibroblasts caused epidermal hyper proliferation in aged mice. In this study, we have generated dermal fibroblast specific N-WASP knockout mice by crossing N-WASP^{fl/fl} mice with Fsp-cre mice (Fig. S1A). The heterozygous, N-WASP^{fl/WT}; Fsp-cre mice from the first generation mice were back crossed to get N-WASP^{fl/fl}; Fsp-cre (N-WASP^{FKO}) mice near to the Mendelian 25% ratio (Fig. S1B). Excision of exon 3 and 4 of (floxed) N-WASP gene was detected in N-WASP^{FKO} mice (Fig. S1C) in tail genomic PCR. Primary dermal fibroblasts isolated from N-WASP^{FKO} mice showed efficient knockdown of N-WASP expression at 13 weeks after birth (Fig. 1A,B). N-WASP^{FKO} mice exhibited a normal life span and no obvious defects were observed in body weight (Fig. S1D), external morphology, reproductive vigor and behavior compared to control littermates (Data not shown). N-WASP expression in keratinocytes has been shown to play critical role in hair follicle cycling via the anti-proliferative TGF β pathway²⁸. Histological analysis of skin layers of N-WASP^{FKO} mice did not show any obvious differences in hair follicle pattern or numbers between weeks 4 to 6 after birth (Fig. S2A). Histological analysis of mice skin taken after 13 weeks or nearly 1 year after birth showed a significant increase in epidermal layer thickness in N-WASP^{FKO} mice compared to either heterozygous (N-WASP^{fl/WT}; Fsp-cre) or control (N-WASP^{fl/fl}) mice (Fig. 1C,D S2B). There was no significant difference in the dermal thickness of mice skin (Fig. 1E). In addition, histological analysis of the ear also showed an enhanced thickening in the N-WASP^{FKO} mice, compared to the control littermates (Data not shown). The thickening of the epidermal layer of N-WASP^{FKO} mice was further characterized by visualizing the nucleus and E-cadherin structures which showed the presence of more than two layers of cells in the epidermis. There was no obvious alteration in the epidermal cell-cell junction, distribution of E-cadherin between control and N-WASP^{FKO} mice (Fig. 2A). In order to verify that the thickening in epidermal layer in N-WASP^{FKO} mice skin is due to a proliferative defect within the epidermis, we performed the immuno-staining of paraffin embedded skin sections for the proliferation marker PCNA. Basal level of PCNA positive cells count revealed that N-WASP^{FKO} mice has significantly higher (40 ± 5 , 13 weeks; 36 ± 10 , 1-year-old) number of PCNA positive keratinocytes compared with control mice (12 ± 3 , 13 weeks; 8 ± 1 , 1-year-old) (Fig. 2B,C). Furthermore, Western blot analysis of whole skin lysate showed higher expression of PCNA in N-WASP^{FKO} mice (Fig. 2D). Thus, suggesting that N-WASP^{FKO} mice have defects in regulating the proliferation of keratinocytes resulting in epidermal hyperproliferation.

N-WASP^{FKO} mice do not have defect in skin barrier function. In order to analyze the skin barrier function we isolated a section of skin from control and N-WASP^{FKO} mice and incubated with Lucifer yellow on the epidermis. The skin section was subsequently analyzed by histological staining to visualize the presence of Lucifer yellow using fluorescent microscopy. In both control and N-WASP^{FKO}, fluorescent dye was restricted to

Figure 1. Enhanced epidermal thickness in N-WASP^{FKO} mice. (A) Genomic PCR analysis of deletion of N-WASP exon 3 and 4 in dermal fibroblasts cells isolated from control and N-WASP^{FKO} mice. (B) Western blot analysis for N-WASP expression of dermal fibroblasts cells isolated 13 weeks old mice. (Note: we achieved greater than 80% pure fibroblasts population from our mice skin fibroblasts isolation method, it contain a little population of other cell types as well). (C) H&E stained dorsal skin sections from 13 weeks and 1 year old mice. Quantification of epidermal (D) and dermal (E) thickness of control and N-WASP^{FKO} mice skin sections. N-WASP^{FKO} mice have more than two epidermal layers compared with control mice. **Represent $p < 0.01$.

the outer layer of epidermis suggesting that there was no obvious defect in the skin barrier function of the epidermis in N-WASP^{FKO} mice (Fig. 3A). The differentiation of keratinocytes in N-WASP^{FKO} and control mice was analyzed by immunofluorescence staining for early and terminal differentiation markers (keratin 10, involucrin, transglutaminase). There were no significant differences in the staining pattern of the differentiation markers between control and N-WASP^{FKO} mice, however there was a thicker layer of cells staining for keratin 10, involucrin and transglutaminase. This suggests that N-WASP deletion in dermal fibroblast cells do not alter epidermal cells differentiation (Fig. 3B).

TPA induced hyperproliferation response. In order to investigate the cutaneous response under the stress condition, we treated the skin of control and N-WASP^{FKO} mice topically with the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA). Expectedly, a single treatment of 6.5 nM/50 μl TPA for 24 hours on dorsal skin resulted in significant thickening of epidermis of both control and N-WASP^{FKO} mice compared with vehicle-treated mice (Fig. 4A,B). Histological analyses of TPA-treated skin sections revealed an elevated hyperproliferative effect in N-WASP^{FKO} mice skin, compared to wildtype littermates. (Fig. 4A), with a concomitant increase in the number of PCNA-positive proliferating keratinocytes in N-WASP^{FKO} mice skin (Fig. 4C). There was no alteration in the epidermal cell-cell junctions identified in stressed induced (TPA-treated) N-WASP^{FKO} mice skin as localization and pattern of E-cadherin was comparable to TPA treated control mice skin (Fig. 4D). Thus, TPA treatment led to enhanced epidermal cell hyperproliferation in N-WASP^{FKO} mice compared to control mice.

Loss of N-WASP expression in dermal fibroblast results in faster wound closure. Dermal fibroblasts play a significant role in wound healing and tissue repair³⁰. In order to determine the effect of N-WASP deficiency in skin fibroblast on tissue repair, we carried out a wound healing assay on 13–15 weeks old control and N-WASP^{FKO} mice. Mice were subjected to full-thickness excisional wounds and the wounded area of mice were photographed and quantified at indicated time points (Fig. 5). N-WASP^{FKO} mice showed a significant faster wound closure at post-wounding days 2, 5 and 7 compared with control mice (Fig. 5A,B). Moreover, significant

Figure 2. N-WASP deficiency in dermal fibroblast enhanced epidermal cell proliferation. (A) Distribution of E-cadherin in control and N-WASP^{FKO} mice was normal but N-WASP^{FKO} mice showed multiple layers of epidermal cells. Red: DAPI (B) Epidermal proliferation measured by PCNA immuno-staining of paraffin embedded dorsal skin sections of 13 weeks and 1 year old control and N-WASP^{FKO} mice. Blue: DAPI (C) Increased number of PCNA positive cells/MF was identified by quantification in N-WASP^{FKO} mice. *Represent $p < 0.05$; *** $p < 0.001$. (D) Western blot analysis for PCNA from whole skin lysate of one year old control and N-WASP^{FKO} mice. N-WASP^{FKO} mice showed enhanced PCNA expression in skin compared with control mice.

reduced wound diameter (shown by arrows) was observed at day 7 in N-WASP^{FKO} mice (Fig. 5C,D). In order to characterize the accelerated wound healing phenotype in N-WASP^{FKO} mice, PCNA staining was performed on skin sections on the 7th day after wounding. We found significantly more number of PCNA positive cells in both dermal and epidermal region in N-WASP^{FKO} mice skin compared to control mice (Fig. 5E). Thus, suggesting that enhanced proliferation rate of dermal/epidermal skin cells after wounding promote faster wound closure in N-WASP^{FKO} mice.

Wound healing involves re-epithelialization as well as wound contraction³¹. Activated keratinocyte proliferate, migrate and subsequently differentiate to form new epithelium while activated fibroblast promote wound healing by wound contraction²⁻⁵. In order to characterize the role of N-WASP in wound contraction, we carried out

Figure 3. N-WASP deletion does not affect skin barrier function. (A) Small freshly isolated sections of skin were analyzed for Lucifer yellow penetration assay. The permeability barrier function was not affected in N-WASP^{FKO} mice. (B) 13 weeks old control and N-WASP^{FKO} mice skin were stained with early (keratin 10, transglutaminase) and terminal (involucrin) differentiation markers. There were no obvious differences in expression of differentiation markers observed between control and N-WASP^{FKO} mice. Red: DAPI.

collagen gel contraction assay. We embedded equal number (2×10^5 cells) of WT or N-WASP^{KO} mouse embryonic fibroblasts (MEFs)²⁵ in Type I collagen matrix. The collagen gel was detached from the side and stimulated with TGFβ1 to measure gel contraction in free floating collagen gel (Fig. 5F). The gel surface area was measured at 24 hr interval for 96 hours. Collagen gel in the absence of fibroblasts or with cells but without TGFβ1 treatment did not contract (note: a slight collagen gel contraction was observed with N-WASP^{KO} MEF cells even without TGFβ1 (Fig. S3C)). In the presence of TGFβ1, collagen gel embedded with N-WASP^{KO} fibroblasts contracted to larger extent compared to embedded with N-WASP^{WT} fibroblasts. (Fig. 5G). Thus, the faster wound healing observed in the N-WASP^{FKO} mice could be due to enhanced wound contraction by the N-WASP deficient fibroblasts.

N-WASP^{FKO} mice have increased fibroblasts and collagen. Wound healing is a multistep process comprising of, the inflammatory phase, proliferation phase and the maturation phase³². In order to determine whether differences in the inflammatory responses could contribute to increased wound healing in N-WASP^{FKO} mice, staining for lymphocytes (CD3/CD4) and neutrophils (Ly-6G antigen) was performed in the granulation tissue. Expression of N-WASP did not significantly alter the ability of wounded tissue to recruit neutrophils, but moderately enhanced the recruitment of lymphocytes (Fig. S4A). Additionally, under basal conditions, the skin of control and N-WASP^{FKO} mice had a very low content of immune cells (data not shown). A cardinal feature

Figure 4. TPA induced hyperproliferation in N-WASP^{FKO} mice. (A) Histological analysis of solvent (acetone) and TPA treated (for 24 hours) dorsal skin of control and N-WASP^{FKO} mice by H & E staining. (B) Quantification of epidermal thickness of acetone and TPA treated dorsal mice skin. N-WASP^{FKO} mice skin is significantly thicker than control mice skin after TPA treatment. (C) Immuno-staining of TPA treated mice skin for analysis of PCNA positive cells. N-WASP^{FKO} mice skin showed significant more number of PCNA positive epidermal cells compared with control mice skin. (D) Analysis of cell-cell junctions by E-cadherin staining in TPA treated mice.

of poorly healing wounds is a persistent inflammatory response at the wound site³³. Despite a slight increase in immune cell infiltration into the wounds of N-WASP^{FKO} mice, the wound closed faster, suggesting that inflammation is unlikely a major contributor to the faster wound healing in N-WASP^{FKO} mouse.

Fibroblasts have been shown to accumulate at the wounded tissue and promote re-epithelialization by producing ECM (collagen) and growth factors³⁴. Although Vimentin is expressed by endothelial cells, it can still be used to identify fibroblast^{35–37}. Immunohistochemistry analysis revealed that N-WASP^{FKO} mice wound had a significant increase in fibroblast number compared to control mice (Fig. 6A). In addition, Western blot analysis also showed increased vimentin expression in N-WASP^{FKO} mice skin (Fig. 6B). Moreover, a moderate increase in vimentin staining was observed in normal N-WASP^{FKO} mice skin (without wounded) compared to control mice skin (Fig. S3A). Dermal fibroblast cells can also differentiate into myofibroblasts and can promote wound healing. Therefore, we analyzed the effect of N-WASP deletion on myofibroblast conversion. N-WASP^{FKO} mice skin showed significant enhanced expression of α -SMA compared to control mice indicating that N-WASP deletion in dermal fibroblast affect myofibroblast differentiation (Fig. 6C). We further evaluated the amount of matrix deposition (collagen content) by Masson's trichrome staining. A moderate increase in collagen content in 7 day wound section was observed in N-WASP^{FKO} mice compared to control mice (Fig. 6D). Intense collagen staining was also observed in N-WASP^{FKO} mice skin (non-wounded) (Fig. S3B). This could be due to the increased number of dermal fibroblast in N-WASP^{FKO} mice skin.

TGF β signaling is increased in N-WASP^{FKO} mice skin. The higher collagen content in the granulation tissue of N-WASP^{FKO} mice suggested the possibility of increased in growth factor secretion. Moreover, crosstalk between fibroblast and keratinocyte occurs through secreted growth factors are essential during wound healing. Members of fibroblast growth factor family (FGFs) have a broad mitogenic spectrum except FGF7/KGF, which is a specific mitogen for epithelial cells³⁸. Analysis of FGF7 expression revealed increase in FGF7 staining in N-WASP^{FKO} mice skin (Fig. 6E). Moreover, moderate increase in pro-inflammatory cytokine IL-1 α was also observed in N-WASP^{FKO} mice skin (Fig. S4B). N-WASP expression in keratinocytes has been shown to regulate TGF β signaling²⁸. Based on this information, we analyzed TGF β signaling using skin lysate from control and N-WASP^{FKO} mice. N-WASP^{FKO} mice expressed higher levels of TGF β -RII, Samd2/3, TAK1, and their respective activated forms (p-Samd2/3, pTGF β -RII) (Fig. 6F, S4C). Our results suggest that deletion of N-WASP expression in dermal fibroblast leads to increase in fibroblasts number, leading to increase in collagen content and secretion

Figure 5. Wound healing is faster in N-WASP^{FKO} mice than control mice. (A) Photographs of wound site at different days after wounding (two wounds for each mouse). (B) Quantification of wound area at different days after wounding. (C) Histomorphometric analysis of wound closure and evaluation of wound closure was performed by measuring the wound diameter of day 0 and day 7 wounds of control versus N-WASP^{FKO} mice (D). N-WASP^{FKO} mice showed a significant decrease in wound diameter compared to control mice. (E) PCNA staining was performed in order to determine the accelerated wound healing in N-WASP^{FKO} mice. Increased PCNA positive cells were observed at the wound edge as well as in the dermal region in N-WASP^{FKO} mice compared to control mice at 7th day after wound. (F) In collagen gel contraction assay, N-WASP^{WT} and N-WASP^{FKO} mouse fibroblast cells were embedded in free floating collagen gel in the presence or absence of TGF-β1 (4 ng/ml). Photographs of collagen gels were taken at 24 hour time interval till 96 hours. The boundaries of the wells were outlined with dotted line. (G) The areas of collagen gel contracted were quantified using ImageJ and scaled using the ruler taken in the images. N-WASP^{FKO} mouse fibroblast cells induced significant faster collagen gel contraction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

of growth factors/cytokines/enhanced TGFβ signaling which results in epidermal hyperproliferation and faster wound healing in N-WASP^{FKO} mice.

Discussion

N-WASP, a member of the WASP family of proteins, regulates the actin polymerization by regulating the activity of the Arp2/3 complex¹⁴. N-WASP is expressed ubiquitously and N-WASP knockout mice were found to be embryonically lethal suggesting that N-WASP is an essential gene in during the development of mice^{24,25}. The N-WASP knockout embryos were found to survive past gastrulation stage and initiate organogenesis but die before embryonic day 12 with neural tube and cardiac defects²⁵. Thus a number of studies have used the *loxP*-Cre system to generate conditional N-WASP knockout mice to study the role of N-WASP in various tissues. Conditional knockout of N-WASP in muscles using *Myf5*-cre and *MyoD*-cre resulted in post-natal death³⁹. Similarly, conditional knockout of N-WASP in brain using *Nestin*-cre also resulted in post-natal death probably due to hydrocephalus²⁶. Two research groups have generated conditional knockout mice with expression of N-WASP ablated in keratinocytes using *K5*-cre^{28,29}, however the two groups reported contrasting roles for N-WASP in keratinocytes. Lefever *et al.* showed that lack N-WASP expression in keratinocytes caused reduced growth of the keratinocytes while Lyubimova *et al.* showed that N-WASP deficiency leads to epidermal hyperproliferation^{28,29}.

We have previously found that N-WASP in fibroblasts plays a critical role in cell-ECM adhesion¹⁵. In order to characterize the role of N-WASP in fibroblasts in the development and maintenance of skin, we generated mice deficient for N-WASP expression in fibroblasts using *Fsp*-cre as the driver. Mice with the genotype of N-WASP^{fl/fl}; *Fsp*-cre, N-WASP^{FKO} were born following Mendelian genetics, and did not show any abnormalities up to 1 year

Figure 6. N-WASP^{FKO} mice has increased number of dermal fibroblasts and FGF7 expression. (A) Fibroblast recruitment was analyzed by vimentin immunostaining of 7 day after wound. The number of fibroblasts were found elevated in N-WASP^{FKO} mice skin. (B) Elevated fibroblast content in N-WASP^{FKO} mice skin was confirmed by western blot for vimentin. (C) Western blot analysis for α-SMA from whole skin lysate of control and N-WASP^{FKO} mice. N-WASP^{FKO} mice showed enhanced α-SMA expression in skin compared with control mice. (D) Collagen content (7 day after wound) was determined by staining with masson's trichrome and found to more in N-WASP^{FKO} mice. (E) Staining with FGF7 antibody, showed elevated expression in N-WASP^{FKO} mice skin. (F) Western blot analysis using GAPDH mAb confirmed that an equal amount of protein was loaded onto each lane. Representative results from three individual animals in each group are shown here. (G) Representative observations of this study: N-WASP deletion in dermal fibroblasts led to enhanced proliferation probably by activation of autocrine/paracrine signaling, which caused increase in secretion/expression of growth factors/cytokines. Thus, resulted in epidermal hyperproliferation and accelerated wound healing.

of growth in the laboratory. There were neither physical abnormalities nor reduced sexual vigor observed in N-WASP^{FKO} mice compared with controls (Data not shown).

Histological sections of the mice skin showed that the N-WASP^{FKO} mice had a thicker epidermis compared with controls and immunostaining for PCNA suggested that the keratinocytes in N-WASP^{FKO} mice were undergoing hyperproliferation (Fig. 2). The fibroblasts and keratinocytes in the skin have a complex interaction in order to maintain homeostasis of the skin and are very critical during wound healing^{31,40}. Thus we characterized the wound healing characteristics of the N-WASP^{FKO} mice and found that N-WASP^{FKO} mice healed the wound faster than that of control mice, the difference was significant at all the time points analyzed (Fig. 5A–D). Conditional knock out N-WASP in keratinocytes led to hyperproliferation however there was no difference in the wound healing characteristics of the knockout mice compared to the control²⁹. Thus the hyperproliferation of keratinocytes seen in N-WASP^{FKO} is probably not the sole reason for faster wound healing in N-WASP^{FKO}. Upon injury the fibroblasts promotes wound healing by several mechanisms^{31,41}, the fibroblasts proliferate, synthesis collagen and differentiate to myofibroblasts which helps in wound healing by contracting the wound^{31,42}. Thus we analyzed the collagen gel contraction properties of WT and N-WASP deficient fibroblasts by TGFβ treatment of cells embedded in collagen matrix. The N-WASP deficient fibroblasts induced a larger gel contraction compared with WT fibroblasts (Fig. 5G). Notably, our finding was in contrast to a previous *in vitro* study that suggested a role of N-WASP in integrating FAK and Arp2/3 signaling to mediate formation of α-SMA-containing cytoplasmic filaments during myofibroblast differentiation and maturation⁴³. In that study, fibroblasts with decreased N-WASP expression (N-WASP knockdown) showed impaired ability to contract collagen gels in response to TGF-β⁴³. The reason for this discrepancy could be due to the difference between the type of cells used. We used knockout cells while Cai *et al.*, used knockdown cells. A number of studies have shown that knockout cells may have different phenotypes to knockdown cells; caveolin-1 knockout cells had higher proliferation than control WT cells while caveolin-1 knockdown (80% reduction) did not exhibit increased proliferation⁴⁴. Expression of

N-WASP was found to be higher in chronic wound compared to acute wound⁴⁵. HaCaT cells (keratinocytes) treated with N-WASP inhibitors (Wiskostatin or 178-1) had increased cell migration. Similarly, knocking down N-WASP expression also caused increased cell migration⁴⁵. Both the chemical inhibitors were found to enhance wound healing in mice, suggesting that inhibition of N-WASP promotes wound healing⁴⁵. The inhibitors are likely to inhibit N-WASP in all cell types thus it is not possible to identify the cell in which N-WASP activity has to be modulated to promote wound healing. Our studies are consistent with the finding that N-WASP inhibition promotes wound healing.

Various growth factors such as fibroblast growth factors (FGFs), platelet derived growth factors (PDGFs) and cytokines play critical roles during wound healing³⁸. PDGFs have been shown to enhance the fibroblast cells proliferation, extracellular matrix production and stimulates fibroblast contraction in collagen matrices⁴⁶. Moreover, impaired wound healing in aged mice is associated with delay in appearance of PDGF receptors⁴⁷. In our study, N-WASP^{FKO} mice skin had increased number of fibroblast compared to control mice as determined by elevated expression of vimentin and increased number vimentin positive cells (Fig. 6A,B). The increased number of fibroblast probably led to enhanced deposition of collagen in N-WASP^{FKO} mice wounded skin tissue (Fig. 6D). A moderate but not significant increase in PDGFR was also observed in N-WASP^{FKO} mice skin (Data not shown). Among FGF family members, FGF7 has been shown to specifically target keratinocytes. FGF7 is secreted by dermal fibroblast and its expression is elevated by injury to skin³⁸. Previous reports suggest that FGF7 induces epidermal cell hyperproliferation and migration without affecting their final differentiation or barrier function, and thus promoted faster healing of wounds⁴⁸. In addition, epidermal atrophy with dermal thickening and delay in re-epithelialization of wounds has been reported in transgenic mice expressing dominant negative FGF7 receptor⁴⁹. Consistent with previous studies, expression of FGF7 was found to be elevated in N-WASP^{FKO} mice skin compared to control mice (Fig. 6E) which probably caused epidermal hyperproliferation (without altering epidermal cell differentiation and barrier function) (Fig. 3) and faster wound healing. Moreover, expression of inflammatory cytokines IL-1 α was found to be upregulated in N-WASP^{FKO} mice skin (Fig. S4B). IL-1 α is secreted by various kinds of cells such as neutrophils, macrophages and fibroblasts. It is very likely that IL-1 α stimulates keratinocytes and fibroblast proliferation and regulates wound healing as topical application of IL-1 α accelerated epidermal wound healing⁵⁰. The relationship between IL-1 α and FGF7 underscores an important epithelial-mesenchymal communication for skin homeostasis. Keratinocytes are known to synthesize IL-1 α in response to injury and IL-1 α has been shown to stimulate fibroblast and keratinocyte growth, collagen synthesis by fibroblasts, and chemotaxis of keratinocytes^{51,52}. In a double paracrine manner, keratinocyte IL-1 α stimulates the expression FGF7 in fibroblasts^{53,54}. The fibroblast FGF7 enhances epidermal proliferation. The deletion of N-WASP in the fibroblast perturbed this epithelial-mesenchymal communication, which manifested as epidermal hyperproliferation and increased fibroblast number.

Fibroblasts produce many components of ECM and are also a relevant source of TGF β ⁵⁵. Our results suggest that N-WASP modulates TGF β signaling in fibroblasts as we found enhanced expression of TGF β signaling proteins in N-WASP^{FKO} mice skin (Fig. 6F, S4C) and N-WASP^{FKO} MEFs caused a slight but significant *in-vitro* collagen gel contraction even in the absence of exogenous TGF β (Fig. S3C). Both WT MEFs and N-WASP^{FKO} MEF caused *in-vitro* collagen gel contraction in the presence of exogenous application of TGF β however the N-WASP^{FKO} MEF caused significantly higher gel contraction (Fig. 5G). TGF β signal through binding to two TGF- β receptors (TGF β -RI and TGF β -RII). TGF β -RII is constitutively phosphorylated and on binding of the ligand, phosphorylates TGF β -RI. Phosphorylation of the receptor complex activates the SMAD intracellular signaling pathway through the receptor Smads (Smad-2 and Smad-3) and co-Smad 4. The receptor SMADs and Smad-4 cross over the nuclear membrane where they regulate a number of genes⁵⁶. In N-WASP^{FKO} mice skin, expression of Smad-2 and Smad-3 and respective phospho-Smad2 and phospho-Smad-3 were elevated (note: we do not observe any difference in ratio of p-Smad3/Smad3 or p-TGF β -RII/TGF β -RII between control and N-WASP^{FKO} mice skin, thus enhanced expression of these TGF β signaling associated factors is probably causing the significant differences in the wound healing between control and N-WASP^{FKO} mice) (Fig. S4C). This enhanced expression of Smad-2 might have up-regulated the expression of receptor TGF β -RII via positive feedback loop and thus representing the increased TGF β signaling in N-WASP^{FKO} mice skin⁵⁶. TGF β is a mediator of tissue repair, wound healing and has been widely implicated in progressive tissue fibrosis⁵⁷. TGF β drives fibroblast-myofibroblast differentiation. Myofibroblasts express α -smooth muscle actin (α -SMA) and are present in granulation tissue where they are responsible for wound contraction. TGF β has been shown to upregulate the expression of α -SMA both *in vitro* and *in vivo*^{58,59}. Conceivably, the enhanced TGF β signaling stimulates fibroblast proliferation and also activates normal fibroblasts to become myofibroblasts. A recent study suggests that reconstitution of vimentin expression in vimentin^{-/-} fibroblasts restored their proliferation and TGF β production, thus is critical for wound healing⁶⁰. This study further potentiates our observation; enhanced vimentin expression/enhanced fibroblasts proliferation/enhanced TGF β signaling. The enhanced TGF β signaling associated factors in N-WASP^{FKO} mice skin probably promotes fibroblasts to myofibroblasts differentiation and faster wound healing. Taken together; our observations suggest that ablation of N-WASP expression in dermal fibroblasts, increase fibroblast cell number, which is probably due to the activation of fibroblasts by autocrine/paracrine signaling. This activation of fibroblasts in N-WASP^{FKO} mice, enhanced expression/secretion of growth factors/cytokine (FGF7/IL-1 α /TGF β signaling) or collagen deposition, resulted in epidermal hyperproliferation and accelerated wound healing in aged mice (Fig. 6G).

Materials and Methods

Animals. N-WASP^{fl/fl} mice were generated as described previously²⁶. In order to generate mouse skin fibroblast cells specific N-WASP expression deletion, N-WASP^{fl/fl} mice were crossed with Fsp-cre (fibroblast specific protein) mice followed by back cross. Genotyping of mice were performed as described previously²⁶. In brief, small piece of tail tips (2–5 mm) were digested in tail digestion buffer containing 50 mM KCl, 10 mM Tris-HCl

(pH 9.0), 0.1% Triton X-100, and 0.4 mg/mL Proteinase K (Sigma-Aldrich; P2308). Tail PCR was performed using KAPA HiFi PCR Kit (KK2101). The primers used to detect flox/flox were (5' to 3' direction); AGCTCAGAGAAGGTGTATTGG (forward) and AGGA CTTACATCTCCAGCAAAGG (reverse). Fsp-cre primers used were: GCGGTC TGGCAGTAAAACTATC (forward), GTGAAACAGCATTGCTGTCACTT (reverse); internal control primers: CTAGGCCACAGAATTGAAAGATC-3' (forward), GTAGGT GGAAATTCTAGCATCATC (reverse). In order to detect the deletion of N-WASP at genomic level, another reserve primer (WASL-Flr) has been used together with flox/flox 5' primer; GGACAGGGTCTATCTCTGAATTCCT. N-WASP^{fl/fl}; Fsp-cre (N-WASP^{FKO}) and control (N-WASP^{fl/fl}) mice were maintained on standard chow diet with unlimited water at constant temperature of 20 °C and 12/12 hours of artificial dark/light cycle. All the experiments were performed according to approved protocols of Institutional Animal Care and Use Committee. All the animal experiments performed were approved by Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University (NTU), (ARF-SBS/NIE-A0250 and ARF-SBS/NIE-A0236).

Histology. Mice were anaesthetized and sacrificed via cervical dislocation. Dorsal skin of mice was flattened on parafilm and fixed in 4% paraformaldehyde overnight in 4 °C. Fixed dorsal skin tissue was then washed with 1X PBS and dehydrated using a different percentage of absolute ethanol solution (70, 80, 90 and 100%) for 1 hour each followed by dehydration in 50% xylene/ethanol mixture and 100% xylene solution. Dehydrated samples were then submerged in paraffin wax at 60 °C overnight and subsequently embedded in paraffin molds. Paraffin embedded tissue was sectioned to 5 µm size on superfrost slides (Fisher). For staining, slides were placed in 60 °C to dissolve the paraffin and subsequently rehydrated and stained with haematoxylin and eosin. Sections were stained with respective primary antibodies; anti-E-cadherin (BD Bioscience; 610181), keratin 10 (sc-23877), involucrin (sc-28558), transglutaminase (sc-25786), KGF7 (sc-1365) and vimentin (550513, BD Biosciences). Fluorescence labeled secondary antibodies (Alexa-488 or 594; Thermo Fisher Scientific) were used at 1:100 dilution. For analysis of proliferating cells, PCNA antibody (sc-56; PC10) was used to stained paraffin embedded dorsal skin sections. Quantification of PCNA positive cells was performed using ImageJ software⁶¹. PCNA positive cells were counted as number of PCNA positive cells per microscopic field. Images were taken using the 40X objective and minimum 6 microscopic fields were counted for each sample for individual experiment and experiment was repeated thrice. Fluorescent images were captured using Olympus microscope fitted with CoolSNAP^{HQ2} camera.

Wound healing assay. 13–15 weeks old control (n = 16) and N-WASP^{FKO} male mice (n = 16) were anesthetized using Ketamine/Xylazine (90 µg/10 µg). Mice were shaved at dorsal side and cleaned with alcohol. Two full thickness circular, (average size of 10 mm in diameter) wounds were made per mouse (separated by at least 1 cm of uninjured skin) using forceps and scissor at dorsal side without injuring the underlying muscle as described^{62,63}. The wounds were covered with occlusive dressing (Tegaderm; 3MTM) in order to prevent any infection. The wounds were photographed with scale using digital camera at 0, 2, 5 and 7th day post injury. The wounded area was determined using imageJ software and wound closure rate was determined as percentage of wound area to the initial wound size. Skin biopsies for histology were performed from each wound at day 0 and 7 after surgery. To visualize collagen, skin sections were stained with masson's trichrome (Cat No: 25088A-F, Polysciences Inc).

TPA induced hyperproliferation. 13–15 weeks old control and N-WASP^{FKO} mice were shaved 24 hours before TPA application (6.5 nM/50 µl in solvent (acetone)) on the dorsal skin. Mice were sacrificed after 24 hours of TPA treatment; skin was isolated, fixed in 4% paraformaldehyde/PBS at 4 °C overnight and embedded in paraffin for histological sections. Sections were stained with haematoxylin and eosin, PCNA and E-cadherin antibodies.

Lucifer yellow penetration assay. For Lucifer yellow penetration assay, 1 mM of Lucifer yellow was poured on to the 1 cm² piece of skin tissue (dorsal skin, epidermal side) with precautions taken as dye was not allowed to go through the dermal side of the skin. After incubation for 1 hour at 37 °C, the skin was washed excessively with PBS and skin was cryo-sectioned. Cryo-sections were stained with DAPI and analyzed with fluorescence microscope.

Collagen gel contraction assay. Experiment was performed as described previously with some modifications⁶⁴. N-WASP^{WT} and N-WASP^{FKO} mouse embryonic fibroblast cells were maintained in DMEM/10% FBS media. N-WASP^{WT} and N-WASP^{FKO} MEFs were kind gift from Scott B Snapper lab²⁵. Mouse embryonic fibroblast cells were trypsinised, counted and 200,000 cells were added into 0.5 mg/ml of Corning Rat Tail Collagen I solution (Corning, USA). 1 µM NaOH was immediately added to neutralize the mixture. The entire mixture was transferred to a 24 well plate immediately. Gel was allowed to set in the 37 °C CO₂ incubator for three hours. After the gel has solidified, complete DMEM was added. Solidified Collagen Gel was detached from the side of the well. Pre-diluted TGFβ1 was added to achieve a final concentration of 4 ng/ml. The complete media was renewed every 24 hours using complete DMEM containing TGFβ1. Image was taken and quantified using ImageJ every 24 hours starting with the 0 hour image. Images were taken by placing the 24-wells plate, containing the gel, above a light box with a ruler alongside the gel as a standard to calibrate the pixel size.

Statistical analysis. For statistical significance analysis, unpaired student's t-test was performed and *p* value < 0.05 was considered as significance. Values in bar charts are the mean ± S.D from three independent experiments.

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Author Contributions

N.J., P.K., K.W.T., and H.B.T.: Maintained the mice and carried out experiments with mice as well as histological sectioning/staining of sections. M.K.S. and J.S.K.C. helped in immunostaining and image capture. N.J.: Drafting of the manuscript. T.T. and N.S.T.: Designed the study, analyzed and interpreted the results and wrote the manuscript.

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Research Article

Comparative study of adipose-derived stem cells and bone marrow-derived stem cells in similar microenvironmental conditions

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ABSTRACT

Mesenchymal stem cells (MSCs), which were first isolated from the bone marrow, are now being extracted from various other tissues in the body, including the adipose tissue. The current study presents systematic evidence of how the adipose tissue-derived stem cells (ASCs) and bone marrow-derived mesenchymal stem cells (Bm-MSCs) behave when cultured in specific pro-adipogenic microenvironments. The cells were first characterized and identified as MSCs in terms of their morphology, phenotypic expression, self-renewal capabilities and multi-lineage potential. Subsequently, the proliferation and gene expression profiles of the cell populations cultured on two-dimensional (2D) adipose tissue extracellular matrix (ECM)-coated tissue culture plastic (TCP) and in three-dimensional (3D) AlgiMatrix[®] microenvironments were analyzed. Overall, it was found that adipogenesis was triggered in both cell populations due to the presence of adipose tissue ECM. However, in 3D microenvironments, ASCs and Bm-MSCs were predisposed to the adipogenic and osteogenic lineages respectively. Overall, findings from this study will contribute to ongoing efforts in adipose tissue engineering as well as provide new insights into the role of the ECM and cues provided by the immediate microenvironment for stem cell differentiation.

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1. Introduction

Mesenchymal stem cells (MSCs) are adult stem cells that are readily isolated from the stroma of several tissues in the adult body and are capable of self-renewal for up to nine passages [1]. These cells also have the ability to differentiate into several cell types, including osteoblasts, adipocytes, chondrocytes and myoblasts *in vitro* [2]. Although the differentiation of MSCs has been an area of much interest to researchers, contradictory evidence regarding the role of MSCs in tissue engineering applications have been observed. Some studies showed that upon differentiation *in vitro*, the MSCs acquired a differentiated phenotype but lacked

functionality of that type of cell [3,4], whereas others have confirmed the full functionality of MSCs *in vitro* upon differentiation [5–8]. Therefore, more research is required in order to reach a consensus.

In terms of the source of MSCs, those derived from the bone marrow are the most extensively characterized and have in fact been used for *in vitro*, preclinical and as well as clinical studies [9–11]. However, the procedure for obtaining the bone marrow is invasive and painful for the donor [12]. In addition, only 0.001–0.1% of the total nucleated cells in the bone marrow are MSCs [13,14]. On the other hand, adipose tissue is considered to be a promising source of MSCs, since these adipose-tissue derived stem cells (ASCs) can be obtained from minimally invasive elective surgical procedures such as liposuction, and a large amount of “waste” tissue can be generated during such procedures [15]. Also, unlike the bone marrow-derived MSCs (Bm-MSCs), approximately 500 fold more stem cells are found in the same amount of starting

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tissue [12,15].

In addition to being a source of stem cells, adipose tissue has also been used as a source of acellular extracellular matrix (ECM) material, mainly in the form of scaffolding material or as a bioactive coating for scaffolds [16–20]. From our earlier studies, we were able to preserve the main proteins and growth factors present in the native adipose tissue during the decellularization process [18,19]. Hence, a coating layer consisting of adipose-derived ECM on a substrate will essentially provide the cells with the relevant biological cues of the native adipose tissue microenvironment.

In general, both ASCs and Bm-MSCs have similar immunophenotypic expression, self-renewal and multi-lineage differentiation potential [21–24]. However some differences between the two cell populations have been reported [5,21,25,26]. For example, ASCs have been shown to possess higher proliferative and angiogenic capabilities *in vitro* as compared to the Bm-MSCs [25,26]. ASCs have also been observed to be genetically more stable with delayed occurrence of senescence [21]. In terms of differentiation potential, some studies have shown enhanced adipogenic differentiation of ASCs [27,28], whilst contradictory findings were reported by others [25,29,30]. Similarly, increased osteogenesis of Bm-MSCs was demonstrated by some [29,31] and opposed by others [25,27]. Overall, there is no conclusive consensus with regards to the differentiation potential of ASCs and Bm-MSCs due to the discrepancies in cell culture conditions and experimental methods. Hence, this study investigates the differentiation potential of these two cell populations into adipocytes when exposed to similar pro-adipogenic microenvironments. A 2D microenvironment is created by growing cells on native adipose ECM-coated tissue culture plastic (TCP) and a 3D environment is created by growing cells within alginate-based scaffolds with mechanical properties matching that of native adipose tissue [32]. Taken together, this study explores the role of the microenvironment in determining the fate of MSCs that are isolated from two different tissues of the body.

To this end, the identities of ASCs and Bm-MSCs were first established *in vitro* and their properties under similar *in vitro* culture conditions were compared. Subsequently, the cells were cultured in both the 2D and 3D microenvironments and the proliferation and gene expression profiles of both the cell populations were compared. Overall, the effect of the cues from these microenvironments on the differentiation potential of the two cell populations (ASCs and Bm-MSCs) was investigated and the findings are reported.

2. Materials and methods

2.1. Isolation and culture of cells

All reagents used for cell culture were purchased from Life Technologies, USA unless otherwise stated. ASCs were isolated from human adipose tissue obtained after elective surgery from National University Hospital, Singapore (DSRB 2011/01721) and Tan Tock Seng Hospital, Singapore (DSRB 2012/00071) following previously established methods [33]. All adipose tissue donors were women with an average age of 42 and all had a BMI of <27 with no history of any chronic viral, autoimmune or metabolic diseases. Bm-MSCs (StemPro[®]) were purchased from Gibco[™] (ThermoFisher Scientific, USA). Three different samples of both the adipose tissue and Bm-MSCs were pooled so as to overcome any biological variation. Both types of cells were cultured *in vitro* using previously established medium composition and culture conditions [18,19,34]. Medium changes were carried out on alternate days and cells were passaged at ~80% confluence. Cells at passage

2–4 were used for the experiments. Cell morphology was visualized under the phase contrast microscope (Primo Vert, Zeiss, Germany) and images were captured using the Zeiss camera and Axio vision software (Zeiss, Germany).

2.2. Cell surface marker expression

The expression of cell surface markers present on the ASCs and the Bm-MSCs was analyzed by flow cytometry following previously established protocols [35]. Briefly, cells were washed with 1X PBS supplemented with 2% FBS. 1×10^5 cells were stained with conjugated monoclonal antibodies, CD73-PE, CD105-PE, CD45-PECy5, CD44-PE and CD29-PE (BD Biosciences, USA) for 1 h at 4 °C. The cells were then washed and resuspended in 1X PBS with 2% FBS. The BD FACSCalibur[™] Flow Cytometer (BD Biosciences, USA) was used to acquire 10,000 events and the resulting data was analyzed using the CELLQuest Software (BD Biosciences, USA).

2.3. Colony forming potential and cellular senescence

The colony forming unit (CFU) assay was performed to check the self-renewal capability of the ASCs and Bm-MSCs. Both cell populations were trypsinized, counted and seeded at a density of 10 cells/cm² and cultured in proliferation medium for 14 days with medium changes on alternate days. On day 14, the colonies were fixed with 4% paraformaldehyde and stained with crystal violet solution (Sigma, USA). All colonies with diameters of more than 2 mm were scored and the total number of colonies were counted [36,37]. The Cellular Senescence Assay Kit (Chemicon, Merck Milipore, Germany) was used for cell populations at passage 9, following manufacturer's instructions. The distinctive blue colour that accumulates in senescent cells due to the activity of senescence-associated β -galactosidase (SA- β -gal) was visualized by light microscopy (Primo Vert, Zeiss, Germany).

2.4. Cellular differentiation

The cells were differentiated into osteogenic, adipogenic and chondrogenic lineages by culturing in proliferation medium enriched with differentiation supplements purchased from Sigma, USA using previously established protocols [33,37]. Successful differentiation of the cells was shown by staining the calcium deposits and lipid globules with Alizarin Red (Sigma, USA) and Oil Red O (Sigma, USA) for the osteo- and adipo-differentiated cells respectively [32,37,38]. The pellet culture of chondro-differentiated cells was embedded in paraffin, sectioned and then stained with Alcian blue (Sigma, USA) to identify the presence of glycosaminoglycans [39]. Briefly, staining was carried out for 15 min at room temperature with 1X PBS washes before and after staining. After air drying, the staining was visualized under a bright field microscope (Primo Vert, Carl Zeiss, Germany).

2.5. Gene expression

The ASCs and Bm-MSCs were used for microarray analysis using the Illumina Bead Array System following previously established procedures [40]. The total RNA was extracted from both ASCs and Bm-MSCs using RNA-Solv (Omega bio-tek, USA) and RNeasy Mini Kit (Qiagen, Netherlands). Amplification was carried out using 500 ng of RNA with the Illumina RNA amplification kit (Ambion[®], USA) according to manufacturer's protocol. The biotin-labelled RNA was then hybridized onto the Illumina Sentrix[®] BeadChip (Illumina, USA). The microarray chip was scanned with an Illumina Bead array reader (Illumina, USA) and data was extracted. Data was normalized using quantile normalization. 3 technical replicates per sample were used. A focus gene list

containing 121 mesenchymal stem cell-related gene candidates was selected and sub-categorized into four analysis groups: (i) Upregulated genes in ASCs ("ASC-upregulated"), (ii) Down-regulated genes in ASCs ("ASC-downregulated"), (iii) Upregulated genes in Bm-MSCs ("Bm-MSC-upregulated") and (iv) Down-regulated genes in Bm-MSCs ("Bm-MSC-downregulated"). Each analysis group was uploaded individually into the Ingenuity Pathway Analysis (IPA) software (Qiagen, USA) for discovery of the associated gene ontologies. The top five enriched ontologies under the categories of "molecular and cellular function" and "physiological system development functions" were considered and ranked according to their respective *p*-values. A lower *p*-value is an indication of a stronger association between the input gene list and the identified gene ontology. In addition, the focus gene list of 121 genes, was also uploaded into the Partek Genomics Suite (Partek Inc., USA) for generation of a heatmap that allows for the visualisation of fold-differences in expression between ASCs and Bm-MSCs.

2.6. Cellular studies on 2D ECM-coated tissue culture plastic (TCP)

Adipose tissue was decellularized in order to remove all cellular content following previously established protocols [18]. Decellularization was confirmed by staining for cells, nucleus and lipid droplets using hematoxylin and eosin, DAPI and Oil Red O respectively. The ECM was characterized by immunostaining for the presence of ECM proteins such as fibronectin, laminin, Collagen I (Col I) and Collagen IV (Col IV). The total protein, collagen and glycosaminoglycan content were also measured. The resulting ECM was dissolved in 0.5 M acetic acid at 4 °C and then diluted to 1 mg/mL concentration in deionized water and adjusted to pH 7.2. The ECM material was then used to coat 24-well tissue culture plates at 37 °C overnight. The tissue culture plates were characterized for successful ECM coating by a change in surface chemistry, roughness, wettability and the presence of ECM proteins. The ECM-coated plates were sterilized by irradiation with ethylene oxide.

ASCs and Bm-MSCs were cultured onto these ECM-coated 24-well plates at a seeding density of 5000 cells/cm² and their proliferation was measured by the PrestoBlue[®] assay (Life Technologies, USA) following previously established protocols [32,37] on days 1, 3, 5 and 7. Briefly, the cells cultured in well plates were incubated with serum-free medium containing 10% PrestoBlue[®] reagent in 5% CO₂ environment at 37 °C. After 30 min, the reagent containing medium was transferred to wells of a 96-well plate and fluorescence was measured at 560/590 nm using a plate reader (Molecular Devices, USA). Fresh proliferation medium was added and the cells were incubated until the next time point.

After 14 days of culture, RNA isolation and cDNA synthesis was carried out for further gene expression analysis by the real time polymerase chain reaction (qPCR) method using the SYBRgreen[®] mastermix (Biorad, USA). Gene expression of adipogenic genes such as peroxisome proliferator activated receptor gamma (PPAR γ), adiponectin and osteogenic genes such as Col I, and osteocalcin were measured. Fold change was calculated by the $\Delta\Delta$ CT method and normalized to housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein L27 and 18s ribosomal RNA. Primer sequences are listed in Supplementary Data, Table S1. The fold change in gene expression for cells grown on 2D uncoated TCP and 2D ECM-coated TCP were compared.

2.7. Cellular studies in 3D AlgiMatrix[®] scaffolds

ASCs and Bm-MSCs were cultured in 3D AlgiMatrix[®] scaffolds (Life Technologies, USA) as per manufacturer's instructions. Briefly, the scaffolds (24-well format) were seeded at a density of 100,000

cells per scaffold in a small volume of 20% firming buffer (FB) in cell culture proliferation medium [32]. The firming buffer allows for post-fabrication tunability and aids in further cross-linking of the scaffolds. The cells were allowed to attach for 45 min at 37 °C and 5%CO₂ conditions before topping up medium. Cell proliferation in the 3D scaffolds was measured using the PrestoBlue[®] assay as mentioned previously on days 1, 3, 5, 7, 10 and 14. On days 7 and 14, the scaffolds were dissolved in 1 mL dissolving buffer (Life Technologies, USA) and centrifuged to pellet the cells that were further used for RNA extraction and subsequently cDNA synthesis for gene expression analysis. qPCR was carried out for gene expression analysis as previously described and the fold change in gene expression for cells in the 3D AlgiMatrix[®] scaffolds was normalized to the gene expression of the cells on 2D uncoated TCP.

2.8. Statistical analysis

All experiments were performed in plural, where cell proliferation (*n*=5), gene expression (*n*=5) and CFU assay (*n*=3) data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using the Mann-Whitney *U* Test for results comparing between the two cell types and between two environments. Statistical significance was assumed where *p* < 0.05.

3. Results

3.1. Characterization of ASCs and Bm-MSCs

The morphology of the ASCs and the Bm-MSCs was visualized under phase contrast microscopy (Fig. 1a) and was found to be similar for both the cell types at various time points. In general, all the cells exhibited typical fibroblast-like morphology consisting of a small cell body with few thin and long cell processes, which was maintained by the cells throughout the *in vitro* culture period. Also, both cell populations were found to be positive for stromal-cell associated markers CD73, CD105, CD44 and CD29 and negative for endothelial cell marker CD45 (Fig. 1b). However, no significant difference was observed in expression levels between both ASCs and Bm-MSCs. In addition, the CFU assay showed that both the cell populations were capable of self-renewal, with ASCs having a significantly (*p* < 0.05) higher number of colonies as compared to Bm-MSCs (Fig. 1c). Approximately 50% of the ASCs seeded formed colonies, whereas only 35% of the Bm-MSCs could do the same. Cellular senescence was observed as a result of the hydrolysis of Xgal by the lysosomal activity of SA- β -Gal, which is visualized as a blue coloration under bright field microscopy. As seen from Fig. 1d (i), by day 14, signs of cellular senescence was present for the Bm-MSCs, which were absent for ASCs (Fig. 1d (ii)). However, cellular senescence was observed for both cell populations on day 21 of culture.

In the current study, differentiation of ASCs and Bm-MSCs was triggered by the addition of respective differentiation supplements to the basal medium. The osteo-differentiated, adipo-differentiated and chondro-differentiated ASCs and Bm-MSCs stained positively for calcium deposits, lipid droplets and glycosaminoglycans with Alizarin Red, Oil Red O and Alcian Blue respectively (Fig. 2). Qualitatively assessed, both cell populations were capable of differentiating into osteocytes, adipocytes and chondrocytes and hence along with the morphology and cell surface marker expression and self renewal abilities, these cells can be successfully identified as MSCs, as defined by the International Society for Cellular Therapy (ISCT) [41].

Fig. 1. Characterization of stem cells (a) Morphology of (i) ASCs and (ii) Bm-MSCs on days 0, 3, 7 and 10 of culture, as visualized by phase contrast microscopy (Scale bar = 100 μ m). Cells from both populations were spindle-shaped with fibroblast-like morphology that was maintained throughout the period of culture. (b) Phenotypic analyses of (i) ASCs and (ii) Bm-MSCs performed by flow cytometry. The expression of stromal cell associated surface markers CD73, CD105, CD44 and CD29 and absence of endothelial cell associated surface marker CD45 was found to be consistent for both the cell populations. (c) Colony forming units (CFUs) of ASCs and Bm-MSCs scored after 14 days of culture at a seeding density of 10 cells/cm². The number of CFUs formed by ASCs were significantly higher than that of the Bm-MSCs within the 14 day period (**p* < 0.05). (d) Cellular senescence of (i) ASCs and (ii) Bm-MSCs observed on day 14 and day 21 of culture. Senescence was observed in ASCs only on day 21, whereas it was already observed in Bm-MSCs on day 14 (Red arrows = blue stain accumulated in senescent cells).

Fig. 2. Microscopic images of (a) ASCs and (b) Bm-MSCs upon staining with (i) Alizarin Red, (ii) Oil Red O and (iii) Alcian Blue for the presence of calcium deposits, lipid accumulation and glycosaminoglycans (GAGs) deposited by successfully differentiated osteoblasts, adipocytes and chondrocytes respectively after 21 days of differentiation in *in vitro* culture (Scale bar=100 μ m). (Black arrows=presence of mineralization, yellow arrows=presence of lipid droplets and blue arrows = presence of GAGs).

3.2. Gene expression studies

Microarray analysis was carried out to further study the expression profiles of ASCs and Bm-MSCs. For this study, out of the 24,350 genes, data from 121 genes that are relevant to the role and function of MSCs were consolidated and analyzed. These include genes related to the ECM (e.g. Collagen type VI), MSCs specific marker genes (e.g. CD44), adipogenic lineage genes (e.g. PPAR γ) and osteogenic lineage genes (e.g. BMP and S100 family of proteins). The focus gene list was segregated into genes that were upregulated and downregulated in both cell populations. A majority of the gene candidates were upregulated in both ASCs and Bm-MSCs (Fig. 3a (i) and (iii)). IPA gene ontology analysis revealed that the self-renewal and migration were the recurring themes associated with the upregulated genes in both these cell populations (Fig. 3a (i) and (iii)). On the other hand, downregulated genes in both ASCs and Bm-MSCs were those that are involved in the molecular and cellular functions of metabolism and biosynthesis (Fig. 3a (ii) and (iv)).

In addition, it was found that the upregulated genes responsible for physiological system development and function (Fig. 3b) were specific to the embryonic, skeletomuscular and neuronal development pathways (Fig. 3b (i) and (iii)), while the down-regulated genes were mainly related to the connective tissue development and organ architecture during the organism's development (Fig. 3b (ii) and (iv)). Taken together, these results suggest that the ASCs and Bm-MSCs are highly similar in terms of their ability to maintain an undifferentiated state *in vitro* (i.e. stemness) and both favour proliferation over specialized metabolic functions, which is in concurrence with earlier characterization results (Figs. 1 and 2).

Notably, some differences were observed for genes down-regulated in ASCs and Bm-MSCs. These included genes associated

with molecular and cellular functions, such as cellular development, cellular functions and maintenance, molecular transport and cell death and survival (Fig. 3a (ii) and (iv)), and those associated with physiological system development and functions such as organismal functions (Fig. 3b (ii) and (iv)).

This is further confirmed from the heat map created from the microarray data (Fig. 4), which showed that, the majority of the genes had similar expression levels in both ASCs and Bm-MSCs. However, some differences between the gene expression profiles of the two cell populations were identified. For example, the expression of Collagen type VI (Col VI) and the S100 calcium binding family of proteins was higher in Bm-MSCs than in ASCs, and the adipogenic lineage genes belonging to the CAAT/Enhancer binding proteins (C/EBP) and the peroxisome proliferator activated receptor (PPAR) family were higher in ASCs.

3.3. Effect of the microenvironment on stem cell behaviour

As shown in Fig. 5a, the presence of the ECM coating did not significantly affect the cell proliferation of ASCs and Bm-MSCs. In fact, the initial attachment of cells (i.e. day 1) on TCP and ECM-coated TCP were found to be similar for both cell populations. However, between the two cell types, the ASCs were found to have an overall higher proliferation rate as compared to the Bm-MSCs. When the gene expression levels of the two cell populations were compared, the fold change in expression levels for both early (PPAR γ) and late (adiponectin) markers of adipogenesis, were found to be significantly ($p < 0.05$) higher in both cell types cultured on the ECM-coated TCP as compared to the uncoated TCP. Furthermore, these markers were also significantly ($p < 0.05$) higher in ASCs as compared to Bm-MSCs regardless of the substrate used for culturing the cells (Fig. 5b). In addition, a significant increase in expression of early Col I was observed for the ASCs in the presence of ECM, but not for the Bm-MSCs. On the other hand, the expression levels of osteocalcin, a late marker of osteogenesis, was found to be similar ($p < 0.05$) in both cell populations and was in fact unaffected by the presence of the adipose tissue ECM coating on the TCP surface.

Similarly, when exposed to the 3D Algimatrix[®] microenvironment, the initial attachment and overall proliferation of ASCs was found to be significantly ($p < 0.05$) higher than the Bm-MSCs, except on day 14, when both the cell populations showed almost equivalent cell numbers (Fig. 6a). In terms of gene expression, the expression of PPAR γ and adiponectin was also significantly ($p < 0.05$) higher in ASCs as compared to the Bm-MSCs (Fig. 6b). However, the expression level of osteocalcin was significantly ($p < 0.05$) higher in Bm-MSCs as compared to the ASCs (Fig. 6b), whilst no significant ($p < 0.05$) difference was observed in the expression of Col I.

4. Discussion

ASCs, isolated from clinical waste in the form of human adipose tissue, were compared to the well-established Bm-MSCs and found to possess characteristic MSC-like properties in terms of morphology, multi-lineage potential and cell surface marker expression. This is in concurrence with previously established data on the cell surface marker expression of MSCs [42–44]. Also, similar to an earlier study carried out [45], both cell populations were found to be capable of self-renewal, with ASCs forming a higher number of colonies as compared to the Bm-MSCs.

When cellular senescence was compared, it was found that at a higher passage (i.e. P9), the Bm-MSCs reached senescence on day 14 whereas the ASCs showed signs of senescence on day 21. In an earlier study [24], Bm-MSCs were also shown to undergo

Fig. 3. Microarray analysis of (i, ii) ASCs and (iii, iv) Bm-MSCs. (a) Ingenuity Pathway Analysis (IPA) of genes related to the molecular and cellular functions that are (i, iii) upregulated and (ii, iv) downregulated during *in vitro* culture of the two cell populations. The five most closely associated gene ontologies are and their number of molecules are represented. (Decreasing *p*-value attributes to stronger associations). (b) Ingenuity Pathway Analysis (IPA) of genes related to the physiological system development and function that are (i, iii) upregulated and (ii, iv) downregulated during *in vitro* culture of the two cell populations. The five most closely associated gene ontologies and their number of molecules are represented. (Decreasing *p*-value attributes to stronger associations).

senescence at an earlier population doubling than the adipose and umbilical cord tissue-derived stem cells from equine tissue. From the current study, our results showed that a similar observation was found for human cells. MSCs cultured *in vitro* are known to undergo cellular aging after long term expansion in culture, which can potentially result in genetic and epigenetic alterations in the cells [24]. Early senescence would therefore result in challenges associated with expansion and proliferation of MSCs for cell-based therapeutic applications. As such, ASCs can be expanded *in vitro* for longer durations than the Bm-MSCs before reaching senescence even at high passages and hence, are recommended for clinical applications.

Microarray analysis was able to provide us with a detailed comparison of gene expression profiles for ASCs and Bm-MSCs. The results from IPA revealed that the two cell populations are very similar cell types displaying characteristics of stemness and differentiation potential. There were subtle differences in the gene expression that correlate well with the phenotypic characterization of ASCs and Bm-MSCs. For example, the gene ontology of “cellular growth and proliferation” was ranked higher in ASCs than the Bm-MSCs, and this is in concurrence with the CFU assay that showed that ASCs had a higher CFU count as compared to the Bm-MSCs. In general, ASCs are capable of performing most, if not all the therapeutic functions of Bm-MSCs and also possess higher self-renewal properties. Hence, in comparison to Bm-MSCs, ASCs make a better source of multi-potent cells owing to their greater abundance and the relative ease of extraction from their native tissue.

Heatmap analysis of the microarray data also showed that the majority of genes had similar expression levels in both ASCs and Bm-MSCs. However, several differences were observed as seen by the change in colour to red (higher expression). For example, Col VI was found to be expressed at higher levels in Bm-MSCs as

compared to the ASCs. Col VI is a flexible protein that contains domains that are shown to bind ECM proteins, which further helps in the organization of the matrix components [46]. Col VI has been known to play an important role in the attachment of the immature hematopoietic cells (HSCs) in the bone marrow microenvironment. As MSCs are stem cells that regulate the niche of the HSCs, Col VI is an indispensable protein secreted by the MSCs for the normal functioning of the bone marrow microenvironment and in bone development and remodeling [47,48]. Furthermore, Col VI is highly expressed in the adipose tissue in conditions of obesity, inflammation and diabetes [49]. Overall, there is a high expression level of Col VI during normal bone development and not in normal adipose development [47,48], which is reflected by the higher expression levels in Bm-MSCs and comparatively lower expression levels in normal ASCs. This further illustrates the point that the same protein can be used by similar type of cells for very different functions depending on their native microenvironments, and that these differences continue to be exhibited even when they are cultured in similar conditions *in vitro*. Hence, even though cellular properties can be affected by long term *in vitro* culture conditions, some inherent niche specific cues do remain active and these play a role in modulating the overall cellular behaviour.

CAAT/Enhancer binding proteins (C/EBP) regulate a number of cell activities including cell proliferation and differentiation in MSCs [50,51]. Amongst the various members of C/EBP, only C/EBP α , β and δ play a major role in early regulation of pre-adipocytes differentiation [52]. The genes of these proteins were also found to be upregulated in ASCs, which suggests that the ASCs had a general predisposition to adipogenic differentiation. The ADFP gene, which is associated with the membrane material of the lipid droplets in adipocytes [53,54], was also found to be expressed at a higher level in ASCs than the Bm-MSCs, thus suggesting a higher possibility of lipid accumulation within the ASCs. PBP is a

Fig. 4. Gene expression levels as measured by microarray analysis depicted as a heat map. The expression levels of genes associated with mesenchymal stem cells, adipogenesis, osteogenesis and extracellular matrix secretion in (a) ASCs and (b) Bm-MSCs are compared. A predisposition to adipogenesis was observed for ASCs, whereas a predisposition to osteogenesis was observed for Bm-MSCs.

Fig. 5. 2D cellular studies results showing (a) Cell proliferation of ASCs and Bm-MSCs cultured on uncoated and ECM-coated TCP as measured by PrestoBlue[®] assay on days 1, 3, 5 and 7. The cell numbers were significantly higher for the ASCs as compared to Bm-MSCs (* $p < 0.05$ signifies significant difference between the cell numbers of ASCs and Bm-MSCs; # $p < 0.05$ signifies significant difference between the cell number of ASCs cultured on uncoated and ECM-coated TCP at the same time point). (b) Gene expression of the ASCs and Bm-MSCs cultured on uncoated and ECM-coated TCP. Expression levels for adipogenic genes (PPAR γ , adiponectin) and osteogenic genes (Col I, osteocalcin) were analyzed and it was found that PPAR γ and adiponectin was significantly upregulated in both cell types when cultured on ECM-coated TCP. Col I expression levels were also upregulated, but only in the ASCs, and no significant difference was found in the expression levels of osteocalcin in both the ASCs and Bm-MSCs (* $p < 0.05$ signifies significant difference between the cells cultured on uncoated and ECM-coated TCP; # $p < 0.05$ signifies significant difference between the ASCs and Bm-MSCs cultured under the same conditions).

binding partner of PPAR γ and in its absence; adipogenesis is inhibited [52]. PBP is also expressed in higher amounts in ASCs as compared to Bm-MSCs, thus reinforcing the adipogenic preference of the ASCs. Taken together, the higher expression of genes associated with adipogenic lineages in undifferentiated ASCs suggests their predisposition to the adipogenesis.

On the other hand, genes of S100A4 and S100A6 calcium binding proteins were expressed in higher amounts in Bm-MSCs

Fig. 6. 3D cellular studies results showing (a) Cell proliferation results for ASCs and Bm-MSCs cultured in 3D AlginateMatrix™ scaffolds. A significant difference between the cell numbers were observed from day 1 to day 10 of culture, however, similar cell numbers were observed on day 14 ($p < 0.05$). (b) Gene expression of the ASCs and Bm-MSCs cultured in the 3D AlginateMatrix™ scaffolds. Upon culturing in basal medium in the 3D environment, ASCs had significantly higher expression levels of adipogenic genes PPAR γ and adiponectin as compared to the Bm-MSCs. On the other hand, significantly higher expression levels of osteocalcin was observed in Bm-MSCs ($p < 0.05$).

than in ASCs. S100A4 plays a role in motility, invasion and tubulin polymerization, whereas S100A6 plays a role in calcium dependent insulin release [55]. These results suggest that the Bm-MSCs have a predisposition to the osteogenic lineage. Overall, from the microarray gene expression results the MSCs cultured in similar conditions *in vitro* appear to possess a predisposition to differentiate into functional cells of the source tissue from where they are initially derived. This demonstrates that the native micro-environment plays a substantial role in influencing the overall cellular fate of the MSC populations.

By culturing the cells on ECM-coated TCP, the intention was to expose both cells populations to a similar microenvironment, in this case, the ECM proteins typically found in adipose tissue. In general, the proliferation of both ASCs and Bm-MSCs did not appear to be affected by the presence of the ECM coating, since the cell numbers were more or less similar for the uncoated TCP wells.

In order to ensure that the ECM-coated plates were indeed coated with ECM proteins, the decellularized adipose tissue ECM was first analyzed and shown to be successfully decellularized with the removal of cellular and lipid content with the retention of the ECM proteins (Supplementary Data, Fig. S1). Successful coating of TCP with this ECM material was then further confirmed by various surface characterization methods prior to subsequent cellular studies (Supplementary Data, Fig. S2).

However, between the two cell populations, the proliferation rate and kinetics were dissimilar, with ASCs growing at a steady rate and to a higher cell number than the Bm-MSCs. This is in concurrence with previous studies that showed that ASCs had a higher proliferation rate than the Bm-MSCs *in vitro* [56]. As there was a significant difference ($p < 0.05$) in cell numbers of ASCs on day 3 between the uncoated and ECM-coated TCP, we postulate that the ASCs recognised the microenvironment as being “native” and therefore proliferated more readily when exposed to the adipose tissue-derived ECM-coated TCP surfaces. The importance of this native microenvironment was further demonstrated when both early and late adipogenic differentiation (*i.e.* PPAR γ and adiponectin expression levels) of ASCs and Bm-MSCs were triggered when cultured on ECM-coated substrates, but not when they were cultured on uncoated TCP. It should be pointed out that this phenomenon took place without the addition of differentiation supplements to the culture medium. Furthermore, the expression levels for Col I was significantly ($p < 0.05$) higher in the ASCs than the Bm-MSCs when cultured in the presence of the ECM coating. Col I, an ECM protein produced during the early events in osteogenesis, is also the major constituent of functionally active adipose tissue [57]. Therefore, the ASCs also produced Col I as a result of adipogenic differentiation [58,59], thus resulting in the higher levels detected. On the other hand, the expression level for osteocalcin, a late osteogenesis marker, was not increased by the presence of the ECM coating in both of the cell types. This further suggests that the adipose tissue ECM provided the cells with tissue specific triggers [60], which then resulted in adipogenesis instead of osteogenesis. In fact, the balance between osteogenesis and adipogenesis of mesenchymal stem cells in the bone marrow is critical in preventing a variety of bone loss conditions [61]. Hence, understanding the role of the ECM in maintaining this balance is crucial for developing therapeutic applications for conditions such as osteoporosis and age-related osteopenia.

When exposed to the 3D alginate microenvironment, the initial cell attachment and proliferation was found to be significantly ($p < 0.05$) different between the two cell populations, with ASCs having a significantly higher ($p < 0.05$) cell number at each time point until day 10 as compared to the Bm-MSCs. However, on day 14, no significant ($p < 0.05$) difference was found between the cell numbers of both populations. Hence, our results showed that in both 2D and 3D *in vitro* cultures, the ASCs inherently grew at a faster and steadier pace than the Bm-MSCs, which is in fact in concurrence with previous studies [56,62]. The morphologies of both types of cells in 2D and 3D conditions were also similar to each other, with fibroblast-like, spindle-shaped morphologies and spherical morphologies observed in 2D and 3D microenvironments respectively (Supplementary Data, Fig. S3). From the gene expression results of the ASCs and Bm-MSCs cultured in the 3D alginate scaffolds, it was found that both the early (PPAR γ) and late (adiponectin) markers of adipogenesis were significantly higher ($p < 0.05$) in ASCs than the Bm-MSCs, which is further confirmation of the adipogenic predisposition of ASCs. In a separate study, the Young's modulus of adipose tissue before decellularization was measured by rheological testing and was found to be in the range of 1.5–3.5 kPa [32]. Hence, the 3D AlginateMatrix[®] scaffolds were considered adipo-mimicking microenvironments as they had similar mechanical properties to that of native tissue [32].

(Supplementary Data, Fig. S4). Interestingly, when cultured in these scaffolds, the Bm-MSCs showed increased incidence of differentiation into the osteogenic lineage, as observed by the significantly ($p < 0.05$) higher expression of late osteogenic marker osteocalcin. This suggests that the 3D microenvironment can trigger the differentiation of stem cells into specific lineages which is greatly influenced by the native source of the cells. Taken together, the mesenchymal stem cells from different sources possess an inherent predisposition to different lineages as defined by their native niche [63]. Thus, the stem cell niche possesses an endogenous control on the residing cells even after they are cultured in different exogenous microenvironments.

5. Conclusion

Overall, this study showed that MSCs derived from the adipose tissue and bone marrow are similar in their characteristics in terms of morphology, phenotypic expression and differentiation potential. However, the ASCs had a higher proliferative and self-renewal capability, as well as delayed senescence as compared to the Bm-MSCs. The difference in proliferation appears to be an inherent property of the cell type and is independent of the microenvironment of the cells, since the same proliferation trend was observed when these cells were cultured on uncoated TCP, adipose tissue ECM-coated TCP or in 3D AlgiMatrix® scaffolds. In addition, adipose tissue-derived ECM was able to trigger adipogenesis in both the cell populations with a higher expression of adipogenic markers in ASCs than the Bm-MSCs. However, in 3D environments, without biological cues from adipose tissue ECM material, the ASCs and Bm-MSCs display predispositions to the adipogenic and osteogenic lineages respectively, suggesting that the micro-environmental factors in 3D adipo-mimicking scaffolds and the inherent predispositions of the cells are both important in defining the ultimate fate of the stem cell. Hence, without the biological cues from the ECM, the mechanical properties of the adipo-mimicking substrates were sufficient enough to trigger spontaneous adipogenesis of ASCs but not Bm-MSCs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.yexcr.2016.09.012>.

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Angiopoietin-like 4 Stimulates STAT3-mediated iNOS Expression and Enhances Angiogenesis to Accelerate Wound Healing in Diabetic Mice

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Impaired wound healing is a major source of morbidity in diabetic patients. Poor outcome has, in part, been related to increased inflammation, poor angiogenesis, and deficiencies in extracellular matrix components. Despite the enormous impact of these chronic wounds, effective therapies are lacking. Here, we showed that the topical application of recombinant matricellular protein angiopoietin-like 4 (ANGPTL4) accelerated wound reepithelialization in diabetic mice, in part, by improving angiogenesis. ANGPTL4 expression is markedly elevated upon normal wound injury. In contrast, ANGPTL4 expression remains low throughout the healing period in diabetic wounds. Exogenous ANGPTL4 modulated several regulatory networks involved in cell migration, angiogenesis, and inflammation, as evidenced by an altered gene expression signature. ANGPTL4 influenced the expression profile of endothelial-specific CD31 in diabetic wounds, returning its profile to that observed in wild-type wounds. We showed ANGPTL4-induced nitric oxide production through an integrin/JAK/STAT3-mediated upregulation of inducible nitric oxide synthase (iNOS) expression in wound epithelia, thus revealing a hitherto unknown mechanism by which ANGPTL4 regulated angiogenesis via keratinocyte-to-endothelial-cell communication. These data show that the replacement of ANGPTL4 may be an effective adjunctive or new therapeutic avenue for treating poor healing wounds. The present finding also confirms that therapeutic angiogenesis remains an attractive treatment modality for diabetic wound healing.

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INTRODUCTION

Impaired wound repair is a major complication of diabetes, resulting in substantial morbidity, lost productivity, and healthcare

expenditures. Furthermore, a poor healing diabetic wound is an open portal for infections, often resulting in chronic inflammation, sepsis, dehiscence, and death. Despite the enormous impact of these chronic wounds, effective therapies remain lacking. Effective management of these problems will require better understanding of the healing process to allow the creation of a salubrious physical and biochemical environment conducive to healing.¹

Normal wound healing proceeds via a continuum of events, including phases of acute inflammation, proliferation, and maturation.² The diabetic state induces alterations of all three of these physiological processes.³ Diabetic wounds are characterized by accumulation of devitalized tissue, increased/prolonged inflammation, poor wound-related angiogenesis, and deficiencies in extracellular matrix (ECM) components.⁴ Diabetic wounds show elevated levels of matrix metalloproteinases and increased proteolytic degradation of ECM components, culminating in a corrupt microenvironment that cannot support healing.^{3,5} Cells such as keratinocytes, fibroblasts, and endothelial cells also display dysregulated expressions of and responses to many growth factors and cytokines. These features typically make diabetic wounds poorly responsive to most treatments, and thus it may be most advantageous to intervene with aggressive strategies that could improve the corrupt extracellular microenvironment. In this respect, the role of matricellular proteins in wound healing is of interest. Matricellular proteins can associate with the diverse proteins of the ECM reservoir, bridging them with cognate cell surface receptors. They reside at the crossroads of cell-matrix communication, modulating several regulatory networks.⁶ Presumably, the regulatory pathways consist of complex networks, creating many opportunities for the compensatory adjustments required for wound repair. Hence, it may be more efficacious to target or replace the necessary matricellular proteins than individual cytokine-mediated candidates.

Angiopoietin-like 4 (ANGPTL4) is a recently identified matricellular protein that is implicated in wound healing.^{7,8} It undergoes proteolytic cleavage after secretion, releasing the N-terminal coiled-coil domain (nANGPTL4) and a C-terminal

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fibrinogen-like domain (cANGPTL4). nANGPTL4 modulates the activity of lipoprotein lipase.⁹ We previously showed that cANGPTL4 facilitates keratinocyte migration and wound reepithelialization. Wounds in ANGPTL4-knockout mice exhibit delayed healing and share many characteristics of diabetic wounds, including reduced expression of matrix proteins, increased inflammation, and impaired wound-related angiogenesis.^{7,8} ANGPTL4 reportedly also has a context-dependent role in angiogenesis and vascular permeability.^{10,11} However, the expression and role of ANGPTL4 in diabetic wound healing remains unclear, and the mechanism by which ANGPTL4 regulates wound angiogenesis is unknown. This study investigated the regulation of wound angiogenesis by ANGPTL4 and examined the potential impact of ANGPTL4 on diabetic wound healing.

RESULTS

Reduced ANGPTL4 expression in impaired diabetic wound healing

First, we characterized the spatiotemporal expression profile of ANGPTL4 messenger ribonucleic acid (mRNA) and protein during the healing of full-thickness excisional splint wounds in ob/+ (normal) and ob/ob (diabetic) mice. Macroscopic

observation on days 7–10 postinjury revealed that wounds of ob/ob mice were closed by 40%, while those of ob/+ mice were completely closed (**Figure 1a** and **Supplementary Figure S1a**). Hematoxylin and eosin staining of day-3, -5, -7, and -10 postwound sections showed impaired epithelial regeneration and formation of granulation tissue in ob/ob compared with ob/+ mice. Histomorphometric analysis of centrally dissected ob/ob wound sections revealed significantly delayed re-epithelialization between days 3–10 postwounding (**Figure 1b**). On day 10 postinjury, the epidermal wound area above the wound bed in ob/ob mice remained larger than in ob/+ mice (**Supplementary Figure S1b**). Quantitative real-time PCR (qPCR) revealed reduced ANGPTL4 mRNA levels at day 3 postwounding in ob/ob compared with ob/+ mice (**Figure 1c**). Consistently, immunoblot analysis showed approximately five-fold reduction in cANGPTL4 protein level at day 5 postwounding (**Figure 1d**), and immunofluorescence staining of ANGPTL4 revealed lower signals in wound epithelia and wound bed in ob/ob compared with ob/+ wounds (**Supplementary Figure S1c**). Similar observations were also obtained from wounds of alloxan-induced diabetic mice (data not shown). Antibody specificity was determined by infrared western blot and immunostaining analysis using skin biopsies from wild-type (ANGPTL4^{+/+})

Figure 1 Expression of ANGPTL4 was reduced in diabetic wounds. **(a)** Photos of normal (ob/+) and diabetic (ob/ob) wound biopsies taken at days 1, 3, 5, 7, and 10 postwounding. Bar = 5 mm. **(b)** Wound closure kinetics of ob/+ and ob/ob mice, with wound surface areas plotted as percentage of day 0 (100%). Data are mean ± SEM, *n* = 12. ****P* < 0.001. **(c,d)** Relative ANGPTL4 mRNA **(c)** and protein **(d)** levels in ob/+ and ob/ob wound biopsies as analyzed by qPCR and immunoblotting, respectively. Graph shows the relative cANGPTL4 protein level compared to in the day 0 wound in ob/+ mice. qPCR values were normalized to the housekeeping gene ribosomal protein L27. β-tubulin served as a loading and transfer control for immunoblotting. Data are mean ± SEM, *n* = 12. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ANGPTL4, angiopoietin-like 4; qPCR, quantitative real-time PCR.

and ANGPTL4-knockout (ANGPTL4^{-/-}) mice (**Supplementary Figure S2a,b**).

Topical application of cANGPTL4 improves the healing rate of diabetic wounds

Next, we examined the effect of a single topical application of recombinant cANGPTL4 on the diabetic wound healing rate. We inflicted two full-thickness excisional splint wounds on the

dorsal skin of ob/ob mice: one to be treated with saline and the other with cANGPTL4 (**Supplementary Figure S3a**). Wound images at days 7 and 10 postinjury showed improved wound closure with cANGPTL4 treatment compared with saline treatment (**Supplementary Figure S3b**). Histomorphological examination of wound sections showed that cANGPTL4 significantly accelerated reepithelialization compared with saline controls, as indicated by the reduced epithelial gap (**Figure 2a**

Figure 2 Topical application of ANGPTL4 improves diabetic wound healing. **(a)** Wound closure kinetics of saline- and cANGPTL4-treated ob/ob wounds, with wound surface areas plotted as percentage of day 0 (100%). Data are mean $\pm$ SEM, $n = 12$. * $P < 0.05$, ** $P < 0.01$. **(b)** Average lengths of neo-epithelium from human skin biopsies cultured for 5 days under normal and diabetic conditions with various cANGPTL4 concentrations. Data are mean $\pm$ SEM, $n = 12$. * $P < 0.05$, ** $P < 0.01$. **(c)** Heat maps show gene expression profiles of ob/+, and saline- and cANGPTL4-treated ob/ob wounds. Genes are clustered according to the following biological gene functions: proliferation, angiogenesis, migration, ECM, apoptosis, and inflammation. The colour spectrum from blue to red depicts the fold-change in LOG form from -1.0 to 1.0. **(d)** Venn diagram comparing total number of genes from ob/+ (blue), and saline- (red) and cANGPTL4-treated (black) ob/ob wounds. Comparisons of the percentages of total numbers of genes changed. ANGPTL4, angiopoietin-like 4.

Figure 3 ANGPTL4 improves wound-related angiogenesis. **(a)** Representative immunoblots and **(b)** graph showing relative protein levels of indicated proteins from ob/+, and saline- and cANGPTL4-treated ob/ob wound biopsies. The graph was determined based on the mean densitometry value of the immunoblot obtained from three independent sets of mice wounding experiments. The data was normalized against the densitometry value of β -tubulin, which served as a loading and transfer control. Data are mean $\pm$ SEM, $n = 12$ mice per set. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ by unpaired Student's t -test. ANGPTL4, angiopoietin-like 4.

and **Supplementary Figure S3c**). Similarly, the length of neopithelia from human skin biopsies was significantly dose-dependently increased in the diabetic-like condition with cANGPTL4 treatment compared to in normal or diabetic conditions without cANGPTL4 (**Figure 2b** and **Supplementary Figure S3d**).

Aberrant growth factor production and activation of cognate signaling cascades contribute to poor diabetic wound healing. To investigate if treatment of diabetic wounds with cANGPTL4 affected their expression profiles, we performed focused qPCR arrays on wound biopsies from control ob/+, and saline- and cANGPTL4-treated ob/ob wounds. We analyzed 77 genes clustered according to the biological functions of proliferation, angiogenesis, cell migration, ECM matrix, cell apoptosis, and inflammation. Heat maps revealed significant expression changes in many genes associated with angiogenesis, ECM remodelling, and inflammation (**Figure 2c**; **Supplementary Tables S2-S4**). cANGPTL4-treated ob/ob wounds exhibited a 61.1% change

in overall differential gene profile compared to saline-treated ob/ob wounds, suggesting an altered wound healing signature (**Figure 2d**).

ANGPTL4 induces angiogenesis in diabetic wounds

Next, we examined expressions of markers of specific cell types, including endothelial cell nuclear antigen (CD31), myofibroblasts (α -smooth muscle actin (α SMA)), proliferating cell nuclear antigen (PCNA), and macrophages (F4/80). Saline-treated ob/ob wounds displayed reduced CD31 and α SMA expressions from day 3 postwounding compared with ob/+ wounds. F4/80 and PCNA expressions were elevated until day 10 postwounding in saline-treated ob/ob wounds compared with ob/+ wounds, consistent with prolonged inflammation and delayed resolution of wound reepithelialization (**Figure 3a,b**). Although the F4/80 and PCNA expression pattern remained unchanged in cANGPTL4-treated ob/ob wounds, the overall expression levels were significantly reduced compared to in saline-treated ob/ob wounds (**Figure 3b**).

Figure 4 ANGPTL4 induced angiogenesis in ob/ob wound. **(a)** Representative immunostaining for CD31 (brown) on ob/+, and saline- and cANGPTL4-treated ob/ob wound biopsies. Bar = 40 μ m. Chalkley's 25 points grids were used to determine the mean chalkley counts shown in the graph for at least three microscopic fields from three independent experiments. Data are mean $\pm$ SEM, $n = 9$. *** $P < 0.001$; **(b)** Representative histograms generated from flow cytometry showing percentage of CD31⁺ endothelial cells population from total cells isolated from ob/+, saline-treated ob/ob and cANGPTL4-treated ob/ob wound biopsies (left panel). Histograms gated from CD31⁺ endothelial cells population showing percentage of Ki67⁺ proliferative cells (right panel). ANGPTL4, angiopoietin-like 4.

Notably, the CD31 and α SMA expression profiles in cANGPTL4-treated ob/ob wounds had reverted to those observed in ob/+ wounds (**Figure 3b**). To further strengthen our finding, immunofluorescence staining of Ki67, α SMA and CD31 were performed on day 5 and day 7 post-wounding biopsies from ob/+, saline- and cANGPTL4-treated ob/ob mice. No difference was observed in number of Ki67-positive proliferating cells among ob/+, saline- and cANGPTL4-treated ob/ob wounds (**Supplementary Figure S4**). Consistent with the finding from western blots, immunostaining of CD31, and α SMA in cANGPTL4-treated ob/ob wounds have reverted to those observed in ob/+ wounds (**Supplementary Figure S4**).

To determine if ANGPTL4 improves wound angiogenesis, we performed immunofluorescence staining of CD31 and quantitative analysis of CD31-positive isolated cells from various treated day-5 wound biopsies. The Chalkley counting method was applied for analyzing the extent of endothelial tube formation wherein a higher Chalkley count represents better angiogenesis. Chalkley counting method is a morphometric point counting systems using microscope eyepiece graticule and has been recommended as a standard in international consensus reports on the quantification of angiogenesis.^{12,13} cANGPTL4 treatment increased CD31 immunohistochemistry staining in ob/ob wounds compared with saline-treated ob/ob wounds (**Figure 4a**, top), indicating increased wound vasculature. The mean Chalkley counts of the cANGPTL4-treated ob/ob wounds was significantly two-fold higher compared to saline-treated ob/ob wounds (**Figure 4a**, bottom). Our fluorescence-activated cell sorting (FACS)

analysis showed $\sim 53\%$ increase in CD31⁺ endothelial cells in cANGPTL4-treated ob/ob wounds compared with saline-treated ob/ob wounds, approaching to the level detected in ob/+ wounds (**Figure 4b**). Furthermore, 72.9% of the CD31⁺ endothelial cell population in cANGPTL4-treated ob/ob wounds also stained positive for proliferation marker Ki67, compared with only 30.7% and 52.4% (CD31⁺Ki67⁺) for saline-treated ob/ob and ob/+ wounds, respectively. Altogether, these observations indicate that cANGPTL4 improves wound angiogenesis.

To check if ANGPTL4 has any direct effect on the endothelial cells (ECs) survival, we have performed FACS analysis for both apoptosis (annexin and PI staining) and proliferation (incorporation of BrdU) assay on attached ECs in the presence and absence of cANGPTL4. Our results did not show any significant difference on apoptosis and proliferation of cANGPTL4-treated ECs compared with saline-treated ECs (**Supplementary Figure S5**).

ANGPTL4 increases nitric oxide production as an angiogenic mediator in diabetic wounds

Our focused gene array analysis revealed that iNOS expression was dramatically increased in ob/ob wounds treated with cANGPTL4 compared with saline. To understand how ANGPTL4 modulates angiogenesis, we first examined the level of nitric oxide (NO), which is a potent angiogenesis mediator in various time points of our wound biopsies. Using a 4,5-diamino-fluorescein (DAF-FM diacetate) -based assay, we detected an overall NO reduction in ob/ob compared with ob/+ wounds (**Figure 5a**). Furthermore, cANGPTL4-treated ob/ob wounds showed significantly increased

NO production from day 3 postwounding compared to those with saline treatment, suggesting that cANGPTL4 may mediate NO production (Figure 5a).

We further found that, in contrast to ob/+ wounds in which endothelial nitric oxide synthase (eNOS) expression peaked at day 5 postwounding, eNOS expression in ob/ob wounds prematurely and transiently peaked at day 3 postwounding (Figure 5b). cANGPTL4 treatment of ob/ob wounds resulted in an increase in eNOS

expression from day 5 postwounding, more closely resembling the expression pattern of ob/+ wounds. The maximal fold-change in eNOS expression was similar between ob/+ and cANGPTL4-treated ob/ob wounds. These observations suggest that the elevation of eNOS in wounds may be a secondary effect. cANGPTL4 also did not modulate eNOS expression in primary human dermal microvascular endothelial cells (Figure 5c). Our results showed that ob/ob wounds expressed little iNOS mRNA compared to

Figure 5 ANGPTL4 regulates NO production in wound epithelium. **(a)** Nitric oxide production in ob/+, and saline- and cANGPTL4-treated ob/ob at indicated postwounding days were determined by measuring the relative DAF-FM diacetate fluorescence level (arbitrary unit, AU). Values were normalized to total protein. **(b–d)** Relative mRNA levels of eNOS **(b)** and iNOS **(d)** from indicated wound biopsies, and from primary human dermal microvascular endothelial cells **(c)**, as analyzed by qPCR. Values were normalized to the housekeeping gene ribosomal protein L27. **(e)** Immunofluorescence staining of iNOS (red) in day 7 wound biopsies of saline- and cANGPTL4-treated ob/ob mice. Dotted lines delineate the epidermis–dermis interface. Bar = 50 μ m. WB, wound bed. **(f)** DAF-FM diacetate fluorescence (green) staining of day 5 postwounding WT and KO mice. Graph shows the mean fluorescence intensity from at least three microscopic fields of three independent mice. Value are mean $\pm$ SEM, $n = 12$. *** $P < 0.001$ compared to WT mice. **(g)** Expression of iNOS mRNA from laser capture microdissected wound epithelium and wound dermis of saline-treated ob/ob mice and cANGPTL4-treated ob/ob mice. mRNA level were normalized to 18s ribosomal RNA. Data are mean $\pm$ SEM, $n = 3$. *** $P < 0.001$ compared to saline-treated ob/ob mice. ANGPTL4, angiopoietin-like 4; DAF-FM, 4,5-diamino-fluorescein; KO, knockout; qPCR, quantitative real-time PCR; WT, wild type.

normal ob/+ wounds, in which iNOS expression peaked at day 3 postinjury. cANGPTL4 treatment in ob/ob wounds increased iNOS expression, which peaked at day 7 postinjury (Figure 5d). Consistently, our immunofluorescence staining for iNOS showed increased intensity in day-7 cANGPTL4-treated wounds compared with saline controls (Figure 5e). To further strengthen our findings, we examined the NO level of ANGPTL4-knockout (KO) mice. Postwounding biopsies from KO mice have significantly lower level of NO detected in the wound epithelium by DAF staining when compared to wild-type (WT) mice (Figure 5f). In addition, we separately examined the mRNA expression of iNOS in laser capture microdissected wound epithelia and dermis from the day 7 postwound biopsies of ob/ob mice (Figure 5g and Supplementary Figure S6). The iNOS mRNA level in the epithelia of cANGPTL4-treated wounds was 10-fold higher than in saline-treated controls. cANGPTL4 treatment of ob/ob wounds did not affect dermal fibroblast iNOS mRNA expression, indicating a cell type-specific response.

To confirm the roles of iNOS and NO induction of angiogenesis and improvement of diabetic wound healing, we cotreated ob/ob wounds with cANGPTL4 and aminoguanidine (AG), a selective iNOS inhibitor. As expected, AG delayed ob/ob wound healing even with cANGPTL4 application, phenocopying saline-treated ob/ob wounds (Figure 6a). Histomorphometric analysis of saline-treated, cANGPTL4-treated, and cANGPTL4 plus AG-treated ob/ob wound sections revealed that the percentage of wound closure at day 7 postwounding was lower in both

saline-treated and cANGPTL4 plus AG-treated ob/ob wound when compared to cANGPTL4-treated ob/ob wound (Figure 6b and Supplementary Figure S7). Altogether, these observations suggested that ANGPTL4 stimulates the expression of iNOS in the wound keratinocytes, elevating NO at the wound site to enhance diabetic wound closure rate.

To further strengthen our findings, we have performed *in vitro* tube formation assay using endothelial cells alone or cocultured with keratinocytes in the presence and absence of cANGPTL4. AG, an iNOS inhibitor, was also used in the assay. The tube formation assays were performed under normal and diabetic conditions. Our observation showed that tubule formation was impaired in diabetic conditions, albeit only slightly. Tube formation was not affected by cANGPTL4, regardless of culture conditions. As expected, AG only has little effect. Notably, our result showed that cANGPTL4-accelerated tubule formation only in the presence of keratinocytes which was inhibited by AG (Supplementary Figure S8). Thus, the tubule-promoting effect of cANGPTL4 works via a keratinocyte-endothelial cells interaction. (Supplementary Figure S8).

ANGPTL4 regulates iNOS expression via STAT3 activation in keratinocytes

To decipher the underlying mechanism of cANGPTL4-mediated acceleration of diabetic wound closure, we first examined the interaction between exogenous cANGPTL4 and integrins in ob/ob wounds using proximity ligation assay (PLA). cANGPTL4 binds to integrin and subsequently activates downstream mediators such as FAK, Src, and ERK to modulate gene expression.⁷ PLA signals, visualized as red spots, were increased in cANGPTL4-treated ob/ob wounds compared to saline-treated wounds, suggesting that cANGPTL4 bound and activated integrin $\beta 1$ (Figure 7a, left panel and Supplementary Figure S9a). This was confirmed using PLA for active integrin $\beta 1$ and active Rac1 interactions, which showed increased PLA signals per cell in cANGPTL4-treated compared with saline-treated ob/ob wounds (Figure 7a, right panel and Supplementary Figure S9b). PLA performed with single specific antibody served as negative controls (Supplementary Figure S9c). Immunoprecipitation using anti-cANGPTL4 followed by immunoblot analysis also showed that the activation of downstream mediators such as phosphorylated FAK and Src (Figure 7b).

In silico analysis of mouse iNOS gene promoter revealed a putative STAT binding site. Immunofluorescence staining showed more phosphorylated STAT3-positive wound keratinocytes in cANGPTL4-treated ob/ob wounds when compared with saline treatment (Figure 7c). Keratin 6 identifies hyperproliferative wound keratinocytes. We also examined the JAK/STAT3 activation in day 5 and 7 post-wound biopsies from ANGPTL4 KO and WT mice. Interestingly, the expression of both phosphorylated STAT3 and JAK1 proteins, i.e. activated proteins, was significantly reduced in KO when compared with WT (Figure 7d). In addition, immunoblotting of phosphorylated STAT3 and JAK1 proteins from laser microdissected wound epithelia of saline- and cANGPTL4-treated ob/ob wounds clearly showed that cANGPTL4 can activate JAK1/STAT3 signaling in the wound keratinocytes (Figure 7e). As expected, this activation of JAK1/STAT3

Figure 6 ANGPTL4 regulates iNOS expression. **(a)** Photographs of ob/ob wounds treated with either saline, cANGPTL4 alone, or cANGPTL4 and aminoguanidine (AG). Representative pictures from three independent experiments from are shown. Bar = 5 mm. **(b)** Box and whisker plot of the percentage of wound closure in saline-treated, cANGPTL4-treated, and cANGPTL4 plus AG-treated ob/ob day 7 postwounds biopsies sections. Histomorphometric measurements were determined from 10 independent sections from the various wound biopsies. Middle bar, median; box, interquartile range; whiskers, full value range; *, possible outlier. ANGPTL4, angiopoietin-like 4.

Figure 7 ANGPTL4 activates JAK1/STAT3 signaling to regulate iNOS expression in keratinocytes. **(a)** Quantification of PLA signals of cANGPTL4:active integrin $\beta 1$ (left panel) and active integrin $\beta 1$:active Rac1 (right panel). **Supplementary Figure S6** presents representative images of all PLA detections. Each PLA signal indicates one detected interaction event. Number of PLA signals per nucleus was determined using Blobfinder (Uppsala, Sweden) software. Data are mean $\pm$ SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$. **(b)** Immunodetection of indicated proteins from anti-cANGPTL4 immunoprecipitates of saline- or cANGPTL4-treated ob/ob wound biopsies. β -tubulin from total tissue lysate was used to verify equal loading. **(c)** Dual immunofluorescence staining of pSTAT3(Y705) (green) and CK6 (red) in saline- and cANGPTL4-treated ob/ob wounds. Dotted lines delineate the epidermis–dermis interface. Nuclei are stained with DAPI (blue). Bar = 40 μ m. WB, wound bed. **(d)** Immunodetection of pSTAT3(Y705), total STAT3, p-JAK1, and total JAK1 in postwounding D5 and D7 of both KO and WT mice. **(e)** Immunoblots of pSTAT3(Y705), total STAT3, p-JAK1, and total JAK1 from laser capture microdissected epithelium of normal human skin, diabetic human skin, saline-treated ob/ob wound, and cANGPTL4-treated ob/ob wound. Data are mean $\pm$ SEM, $n = 3$. **(f)** Representative histograms generated from flow cytometry showing the mean intensity of DAF-FM signal from STAT3 knockdown keratinocytes in the absence or presence of cANGPTL4. Data are mean $\pm$ SEM, $n = 3$. **(g)** Relative mRNA expression level of iNOS from STAT3 knockdown keratinocytes in the absence or presence of cANGPTL4, $n = 3$. ** $P < 0.01$, *** $P < 0.001$. **(h)** ChIP assays were conducted using preimmune IgG or antibodies against pSTAT3(Y705) in saline- (U) and cANGPTL4-treated (T) ob/ob wounds at day 7 postinjury. Graph (right panel) shows quantitative ChIP as percentage of input. Specific regions spanning promoter binding sites of the *iNOS* gene were amplified using appropriate primers (**Supplementary Table S1**). A control region served as a negative control. ANGPTL4, angiopoietin-like 4; ChIP, chromatin immunoprecipitation; DAF-FM, 4,5-diamino-fluorescein; KO, knockout; PLA, proximity ligation assay; WT, wild type.

Figure 8 Schematic illustration of the autocrine and paracrine effect of ANGPTL4 on cutaneous wound healing. cANGPTL4 can interact with integrin $\beta 1$, and activate the Src, ERK, and AKT signaling cascades, leading to subsequent JAK1/STAT3 activation. Activated STAT3 can then mediate the upregulation of iNOS expression by specifically binding to the cognate responsive elements in the promoter of *iNOS* gene. Wound angiogenesis can then be promoted by the increase in NO production from the wound keratinocytes in the wound microenvironment. ANGPTL4, angiopoietin-like 4; PLA, proximity ligation assay.

signaling was also observed in laser microdissected wound epithelium of normal human skin compared to diabetic human skin (**Figure 7e** and **Supplementary Figure S6**). To determine whether cANGPTL4 regulates iNOS expression via STAT3 signaling in keratinocytes, we measured iNOS mRNA by qPCR and NO production using DAF-FM in STAT3-knockdown keratinocytes (Ker_{STAT3}) treated with cANGPTL4. Our result showed reduced iNOS mRNA and NO levels in Ker_{STAT3} compared with Ker_{CTRL} (**Figure 7f,g**).

Finally, *in vivo* chromatin immunoprecipitation showed that phospho-STAT3 specifically bound to the cognate responsive elements in the promoter of the mouse *iNOS* gene in ANGPTL4-treated but not saline-treated ob/ob wounds (**Figure 7h**, left panel). No immunoprecipitation or amplification was seen with preimmune IgG or with a control sequence upstream of the responsive elements in the promoter *iNOS* gene (**Figure 7h**, left panel). Quantitative ChIP showed ~15-fold enrichment in STAT-binding region (SBE) when compared with cognate controls (**Figure 7h**, right panel). Altogether, these observations indicate that ANGPTL4 stimulates NO generation in wound keratinocytes through direct transcriptional regulation of the *iNOS* gene by pSTAT3.

DISCUSSION

Diabetic wounds heal poorly, often resulting in ulceration. Relatively few topical medications are efficacious for ulcer care,

and amputation rates remain high in nonhealing diabetic patients. Growing evidence indicates that altered expressions of cytokines and ECM proteins play significant roles in the delayed healing of diabetic wounds. Experimental evidence shows that vascular-endothelial growth factor, platelet-derived growth factor, and epidermal growth factor accelerate diabetic wound healing in animal models^{14–16}; however, successful results require high doses of these growth factors. Furthermore, these therapeutic strategies ignore the central coordinating role of the matrix proteins in orchestrating and enhancing the cascade of cellular events in the wound healing response.

Here, we showed that the topical application of recombinant cANGPTL4 accelerated wound reepithelialization in diabetic mice by improving wound angiogenesis. The matricellular protein cANGPTL4 expression has been weakly detected in normal intact skin and is markedly elevated upon wound injury. In contrast, cANGPTL4 expression remains low throughout the healing period in diabetic wounds. Topical application of cANGPTL4 modulates several regulatory networks involved in cell migration, angiogenesis and inflammation, as evidenced by an altered wound healing gene expression signature when compared with saline-treated diabetic wounds.

Various studies have suggested that the role of ANGPTL4 in angiogenesis may be context-dependent. Conflicting roles for ANGPTL4 in tumor vascularization have been reported.^{17,18} In a model of oxygen-induced retinopathy, pathological neovascularization was strongly inhibited in ANGPTL4-deficient mice.¹⁹ A recent study showed that ANGPTL4 improves the cerebral vasculature network after brain injury and displays vasculoprotective properties when used as an adjunct to vascular-endothelial growth factor.²⁰ Evidence from ANGPTL4-knockout mice indicates that ANGPTL4 is proangiogenic in normal wound healing.⁷ These observations suggest a role for ANGPTL4 in angiogenesis, although the underlying mechanism remains unresolved. Here, we showed that ANGPTL4 has a dramatic influence in the expression profile of endothelia-specific CD31 in diabetic wounds, returning its profile to that observed in wild-type wounds. cANGPTL4 induces NO production through an integrin/JAK/STAT3-mediated upregulation of iNOS expression in diabetic skin, thus revealing a hitherto unknown mechanism by which ANGPTL4 regulates angiogenesis via keratinocyte-to-endothelial-cell communication (**Figure 8**). Interestingly, impaired wound angiogenesis was also observed in mice that lack $\alpha 3\beta 1$ integrin in epidermis, lending support for a keratinocyte-to-endothelial-cell crosstalk that is dependent on epithelial integrin-mediated signaling.²¹ PLA and coimmunoprecipitation analysis revealed that exogenous cANGPTL4 interacted with integrin $\beta 1$. ANGPTL4-bound integrins activated downstream mediators such as Rac1 and JAK. We further showed that ANGPTL4 regulates iNOS expression via STAT3 activation in keratinocytes as evidenced by immunoblot analysis of microdissected wound epithelia, STAT3-knockdown keratinocytes and *in vivo* chromatin immunoprecipitation. Vascular-endothelial growth factor is a potent angiogenic factor that can also modulate NO generation via eNOS upregulation.²² In contrast to vascular-endothelial growth factor, cANGPTL4 had little effect on eNOS expression, but clearly induced iNOS expression. We observed that ANGPTL4 treatment shifted

the eNOS expression peak to day 7 postwounding, which is most likely due to the increase in proliferating endothelial cells (Ki67⁺CD31⁺) already detected in the day-5 wounds. Numerous studies have shown that NO can profoundly impact angiogenesis, and we are only beginning to understand the mechanism for its observed beneficial effect on wound repair.^{23,24}

Inflammation plays both positive and negative roles in cutaneous repair—the level and length of inflammation can dictate both the healing time and quality of repair.²⁵ Recapitulating the dampened inflammatory responses found in foetal and adult chronic wounds has proved useful in accelerating repair and reduced scarring.^{26,27} We observed that expression of the macrophage marker F4/80 was reduced upon treatment with cANGPTL4 compared to saline, suggesting that cANGPTL4 may reduce wound inflammation. The relationship between ANGPTL4 and inflammation remains unclear. ANGPTL4 has been implicated in other inflammation-associated pathologies; it plays a protective role against the severe proinflammatory effects of saturated fat, as well as in arthritis (particularly in models dependent on adaptive immunity) and in atherosclerosis. The underlying mechanism is unknown and is likely to be highly complex, beyond the scope of this current work.

We have proposed that cANGPTL4 be topically applied to diabetic wounds, which limits systemic circulation and therefore reduces unspecific effects on other tissues and organs. The poorly vascularized state of diabetic wounds further restricts the range of effect of topical cANGPTL4 application. The expression of cANGPTL4 was strongly elevated during normal wound healing, but was absent in the diabetic condition. Hence, our treatment seeks to restore physiological levels of cANGPTL4 functions in diabetic wounds using exogenous recombinant cANGPTL4 proteins. Indeed, the topical application of cANGPTL4 to the wound bed restore expression profile of many crucial wound healing mediators in diabetic wounds closer to that of normal wounds. Our study suggests that the replacement of matricellular protein ANGPTL4 can provide an adjunctive or new therapeutic avenue for treating poor healing wounds such as diabetes-associated ulcers. Furthermore, the present results confirm that therapeutic angiogenesis remains an attractive treatment modality for diabetic wound healing.

MATERIALS AND METHODS

Reagents. We used the following antibodies: Ki67 (NCL-Ki67p) and keratin 6 (NCL-CK6; NovoCastra, Leica Biosystems, Wetzlar, Germany); β -tubulin (H-235, sc-9104), PCNA (PC10, sc-56), α SMA (α -SM1, sc-130617), and goat antirabbit and antimouse IgG-HRP (Santa Cruz Biotechnology, Dallas, TX); CD31 (BD Pharmingen, San Jose, CA); CD68 (FA-11; Biolegend, San Diego, CA); F4/80 (AbD Serotec, Raleigh, NC); STAT3 (ab7966; Abcam, Cambridge, UK); iNOS/NOS II (06-573; Merck Millipore, Billerica, MA); pStat3(Tyr705; #9145; Cell Signaling Technology, Danvers, MA); ID3 (2B11, MA1-23242; Thermo Scientific, Waltham, MA). cANGPTL4 monoclonal antibodies were produced in-house (clone 4A11H5 and 3F4F5). All secondary Alexa Fluor antibodies were from Molecular probes. We used the diaminobenzidine peroxidase substrate kit (Vector Laboratories, Peterborough, UK). Unless mentioned otherwise, all chemicals were from Sigma-Aldrich and molecular biology enzymes from Fermentas. All oligonucleotides were synthesized by Sigma-Proligo (Sigma-Aldrich, Singapore).

Expression and purification of recombinant cANGPTL4. The expression and purification of recombinant cANGPTL4 protein was performed in

Drosophila S2 cells as described in our previous publication.⁸ Recombinant ANGPTL4 proteins were purified from the conditioned medium of stable ANGPTL4-expressing S2 cells by preparative isoelectric membrane electrophoresis.

Animals and wounding experiments. All animal studies were approved and performed in compliance with the regulations of the Institutional Animal Care and Use Committee of Nanyang Technological University. The ANGPTL4-KO mice strain was previously described.²⁸ Male mice aged 6–8 weeks were used in this study and were individually caged during the wounding experiments. Mice were anesthetized prior to surgery by a single intraperitoneal injection of 80 mg/kg ketamine/10 mg/kg xylazine. Two circular 5-mm full-thickness excisional splint wounds were created, as previously described.²⁹ On the day of surgery (day 1), 50 μ l of recombinant cANGPTL4 protein (1 mg/ml) or PBS in 4% carboxyl methylcellulose (CMC) was topically applied to the wounds and protected with an occlusive dressing (Tegaderm; 3M, Singapore). Mice were euthanized by CO₂ inhalation on days 1, 3, 5, 7, and 10 postwounding. Wound biopsies were subjected to photographic imaging, histomorphometric, and biological analyses. Wound biopsies were processed and wound surface area was determined as previously described.^{26,29} Surface wound area at each time-point was expressed as a percentage of initial wound area at day 1 (100%). Histomorphometric measurements of sections were performed as previously described.^{7,29}

In vitro diabetic wound assay. Human skin tissue samples were provided by Dr. Marcus Wong from Plastic, Reconstructive, and Aesthetic Surgery of Tan Tock Seng Hospital, Singapore under an ethical committee review board approval with reference no. (NHG DSRB Ref: 2012/00071). All human samples had been deidentified subcutaneous adipose was removed. Skin biopsies were made using a 4-mm biopsy punch, and were cultured in DMEM supplemented with 10% foetal bovine serum containing either 5.5 mmol/l glucose and 1 nmol/l insulin (normal condition) or 25 mmol/l glucose and 10 nmol/l insulin (diabetic condition) in a humidified incubator with 5% CO₂ at 37 °C.³⁰

Assessment of wound healing. Wounds were photographed at days 1, 3, 5, 7, and 10 postwounding, using a Canon G12 digital camera. Each image included a ruler to allow standard calibration of measurements. Surface wound area was quantified at each time-point, using Image-Pro Plus version 5.1.0.20 software (Media Cybernetics), and was expressed as a percentage of initial wound area at day 1 (100%). Histomorphometric measurements of sections were made through the centre of the wound. Sections of wound biopsies were stained with hematoxylin-eosin. Histological images were visualized with Nikon Eclipse 90i brightfield microscope using a Plan Fluor, 10 $\times$ /0.30 objective and QCapture Pro version 5.0.1.26 software (QImaging, Canada). The measurements were performed three times from random sections using Adobe Photoshop CS5.1, and the image pixels were calibrated to micrometer using the scale bar.

Laser capture microdissection. Paraffin-embedded sections were mounted onto MembraneSlides 1.0 PEN (Carl Zeiss, Oberkochen, Germany) that was pretreated with UV for 30 minutes. Hematoxylin- and eosin-stained sections were then subjected to laser capture microdissection (LCM) using Arcturus XT laser capture microdissection system according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). LCM tissues were collected onto an adhesive CapSure Macro LCM Caps (Life technologies).

Immunoblot and western blot analysis for LCM samples. The LCM caps with the captured samples were capped onto 500 μ l centrifuging tubes containing 15 μ l of lysis buffer and process as previously described.³¹ Total RNA was isolated using RecoverAll total nucleic acid isolation (Ambion, Life Technologies) according to the manufacturer protocol. Fifty nanograms of RNA were subjected to Full Spectrum Complete Transcriptome

RNA Amplification (System Biosciences, Mountain View, CA) according to manufacturer's recommendation prior qPCR.

Immunohistochemistry for CD31. Sections were fixed, processed, and blocked as for immunofluorescence staining. Sections were incubated overnight at 4 °C with rat anti-mouse CD31 antibody, followed by incubation with biotinylated goat anti-rat IgG antibody (Vector laboratories). After incubation with secondary antibodies, sections were incubated in avidin:biotinylated enzyme complex (ABC; Vector Laboratories) for 30 minutes. Sections were developed with 3,3'-diaminobenzidine substrate (Vector Laboratories), which produced a brown stain. Slides were mounted with Fluka Eukitt quick-hardening mounting medium (Sigma-Aldrich). Negative controls did not include the primary antibody. Images were taken using Carl Zeiss MIRAX MIDI using a Plan-Apochromat 20×/0.8 NA objective and Marlin F146.C camera, and MIRAX Viewer Version 1.11.49.0 software (Carl Zeiss Microimaging GmbH, Oberkochen, Germany). Chalkley counting procedure was used for the quantification of capillaries in the wound bed as previously described.^{12,13,32,33} Briefly, the three most vascular areas (hot spots) with the highest number of microvessel profiles were chosen subjectively from each wound biopsies. A 25-point Chalkley grid was applied to each area and oriented to permit the maximum number of "hit on" points. The mean Chalkley count was determined.

Chromatin immunoprecipitation. Briefly, biopsies from saline- and cANGPT4-treated ob/ob wounds at day 7 postinjury were cut into small pieces and then digested with 0.5% collagenase I at 37 °C. Cells were retrieved and cross-linked using 0.5% formaldehyde for 10 minutes at 37 °C, followed by sonication in sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10 mmol/l ethylenediaminetetraacetic acid, and 50 mmol/l Tris-HCl, pH 8.1) to obtain cross-linked DNA of 200–500 bp in length. Of the supernatant, 10% was used as input, while the remaining amount was used to for complexes with pSTAT3(Y705) specific antibody (Cell Signaling Technology) or preimmune IgG. Complexes were pulled down by Protein A/G (Santa Cruz) and were eluted with elution buffer (1% SDS, 0.1 mol/l NaHCO₃). DNA fragments were reverse cross-linked at 65 °C for 6 hours. PCR was performed with 5–10 µl of the eluate, using primers flanking either the STAT-binding site of the iNOS gene (–942 to –934) or an unrelated control sequence (>2 kbp away from the binding site). The primer pairs used are presented in **Supplementary Table S1**.

Immunoblotting. Wound biopsies were homogenized with ice-cold protein lysis buffer (20 mmol/l Na₂H₂PO₄, 250 mmol/l NaCl, 1% Triton X-100, 0.1% SDS, and 1 mmol/l phenylmethanesulfonyl fluoride; pH 8.0). Total protein lysates were resolved using 10% SDS-PAGE and electrophoresed onto Immobilon-FL polyvinylidene fluoride membrane (IPFL00010, Merck Millipore). Membranes were blocked with 1× Odyssey Blocking buffer (#927–40000, LI-COR Biotechnology, USA) for 1 hour at room temperature. The membrane was then incubated overnight at 4 °C with the indicated primary antibodies in 0.5× Odyssey Blocking buffer diluted with TBST (50 mmol/l Tris HCl, pH 7.6, 150 mmol/l NaCl, and 0.05% Tween-20). Membranes were washed thrice with tris-buffered saline with Tween 20 (TBST), and incubated with appropriate IRDye680- or 800-conjugated anti-IgG secondary antibodies (1:10,000, LI-COR Biotechnology) for 1 hour at room temperature. Protein bands were revealed using the Odyssey CLx Infrared Imaging System, and signals were quantified using ImageStudio Software (LI-COR Biotechnology). To detect cANGPTL4, the membrane was blocked overnight at 4 °C with 1× Odyssey Blocking buffer. The membrane was then incubated overnight at 4 °C with anti-cANGPTL4 mouse monoclonal antibodies (clone 4A11H5) at 0.6 µg/cm² of the membrane in 0.5× Odyssey Blocking buffer diluted with TBST.

RNA extraction and reverse transcription. Total RNA was extracted using TRIzol Reagent followed by the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) according to the manufacturer's

protocol. Total RNA was quantified based on the A260/A280 absorbance using the Nanodrop ND1000 (Thermo scientific). Total RNA was reversed transcribed using iScript Reverse Transcription Supermix for RT-1PCR (#170–8841, Bio-Rad).

Focused real-time PCR array. Real-time PCR arrays were used to analyze the expression of a focused panel of genes. qPCR was performed with the CFX96 real-time PCR detection system (Bio-Rad), using KAPA SYBR qPCR Universal Master Mix (KAPABiosystems, Wilmington, MA). Melt curve analysis was included to ensure that only one PCR product was formed. Primers were designed to generate a PCR amplification product of 100–250 bp. Only primer pairs yielding unique amplification products without primer-dimer formation were subsequently used for real-time PCR assays. Expression was normalized to that of the housekeeping gene ribosomal protein L27, which did not change under any of the studied experimental conditions. The qPCR primer sequences are available in **Supplementary Table S1**.

Hydroxyproline assay. Wound biopsies were frozen in liquid nitrogen, and then thoroughly homogenized in distilled water. Aliquots of samples (50 µl) were hydrolyzed in 2 N NaOH at 120 °C for 2 hours, and then oxidized with chloramine-T reagent (0.0127 g/ml) for 25 minutes at room temperature. The chromophore was then developed with the addition of *p*-dimethylaminobenzaldehyde reagent (0.3 g/ml dissolved in methanol/hydrochloric acid solution, 2:1 v/v). The absorbance of the formed red-dish-hue complex was measured at 550 nm using a SpectraMax M2e Multi-Mode Microplate Reader and SoftMax Pro Microplate Data Acquisition & Analysis Software (Molecular Devices). Trans-4-hydroxy-L-proline (0–300 µg/ml) was included as a standard. The value of unknown hydroxyproline was determined from the standard curve. Weight of wound biopsies was used for normalization.

NO determination. Intracellular level of NO from wound biopsies were measured using cell-permeable DAF-FM diacetate (D23844, Molecular probes, Invitrogen). Wound biopsies were lysed in Krebs buffer, and incubated with 10 µmol/l DAF-FM diacetate for 30 minutes at 37 °C in darkness. We immediately recorded the fluorophore signal at 495 nm excitation and 515 nm emission wavelengths using a GloMax 20/20 Luminometer (Promega). Fluorescence was expressed as arbitrary fluorescence units, and was measured with the same instrument settings for all experiments.

Intracellular staining and flow cytometry. For determination of proliferative endothelial cells, intracellular staining for CD31 and Ki67 was carried out on the cells isolated from fresh wound biopsy. Cells were fixed and permeabilized in 300 µl of cytoperm buffer (0.01% paraformaldehyde with 0.1% NP40 in PBS) for 20 minutes on ice and blocked with 10% normal goat serum in PBS for 30 minutes at room temperature. Following that the cells were incubated with staining buffer (3% NGS in PBS) containing the rat anti-CD31 (1:200) and rabbit anti-Ki67 (1:200) for 30 minutes on ice, followed by incubation with FITC conjugated goat antirat IgG and PE conjugated goat antirabbit IgG secondary antibody for another 30 minutes on ice before flow cytometric analysis. For determination of NO, intracellular staining with cell-permeable DAF-FM diacetate (D23844, Molecular probes, Invitrogen) was carried out on the transfected keratinocytes. Cells were incubated with in DMEM alone phenol red free containing 2 mmol/l of DAF-FM diacetate for 30 minutes in a humidified incubator with 5% CO₂ at 37 °C. Cells were then trypsinized and resuspended with DMEM supplemented with 10% fetal bovine serum phenol red free before flow cytometric analysis. Flow cytometry was carried out using BD Accuri C6 flow cytometer (BD Biosciences) and data analysis was performed using Flowjo software version 7.6.5 (Tree Star).

Transient suppression of STAT3 in keratinocytes. Keratinocytes were seeded at 5 × 10⁴ cells in 6-well culture plates and were incubated overnight in a humidified incubator with 5% CO₂ at 37 °C prior to the

transfection assay. The experimental setups include untreated cells, non-targeting scrambled siRNA and ON-TARGETplus SMARTpool STAT3 siRNA (L-003544-00-0005; Thermo scientific). A final concentration of 5 µmol/l of siRNA and 4 µl DharmaFECT transfection reagent with serum free medium was added to each seeded well according to the manufacture protocol. Transfection medium was replaced with fresh media 16 hours after the transfection and analysis were performed within 24–96-hour posttransfection.

In vitro endothelial tube formation assay. Keratinocytes was seeded onto the 3.0 µm transwell inserts with a final density of 4×10^5 cells/insert. The 24-well plates were coated with 100 µl of Matrigel (Becton Dickinson, Bedford, MA). Primary human umbilical vein endothelial cells of <5 passages were then plated in duplicate at a concentration of 4×10^4 cells/well in 400 µl of endogrow (endothelial cell growth media) medium. Tubule formation was monitored by liver imaging.

Statistical analysis. Statistical analyses were performed using two-tailed Mann-Whitney tests or two-way ANOVA using SPSS v.19 software (IBM Corporation). A *P* value of <0.05 was considered significant.

SUPPLEMENTARY MATERIAL

Figure S1. Histomorphometric analysis of centrally dissected ob/+ and ob/ob wound sections.

Figure S2. Specificity of monoclonal antibodies against mouse ANGPTL4.

Figure S3. cANGPTL4 improves diabetic wound healing.

Figure S4. Immunofluorescence staining of wound biopsies.

Figure S5. ANGPTL4 has no direct effect on endothelial cells survival and proliferation.

Figure S6. Laser capture microdissection (LCM) of skin specimens.

Figure S7. Aminoguanidine inhibits wound healing by cANGPTL4.

Figure S8. Effect of ANGPTL4 on *in vitro* endothelial tubule formation.

Figure S9. ANGPTL4 activates the integrin signaling pathway.

Table S1. List of primer pairs sequences.

Table S2. Gene expression in ob/+ wound.

Table S3. Gene expression in Saline-treated ob/ob wound.

Table S4. Gene expression in cANGPTL4-treated ob/ob wound.

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Review

Probing for protein-protein interactions during cell migration: limitations and challenges

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Summary. Cellular migration is a fundamental biological process occurring as early as embryogenesis to the pathological state of cancer metastasis. Nearly all cellular migrations involve an extracellular signal that is transduced internally by members of a signalling cascade. These signal transduction events are driven by protein-protein interactions (PPIs) that coordinate intracellular activities to enable a cell to migrate. Understanding these PPIs will provide valuable insight into how cellular migration can be modulated perhaps towards a therapeutic end. Histologically, not many techniques are available to demonstrate PPIs. Contrasting agents only demonstrate the presence of a particular protein, and perhaps its co-localisation with another protein. Yet, co-localisation need not necessarily indicate physical interaction between the two proteins. In this review, we highlight four commonly used methods that continue to expand our understanding of PPIs underlying cell migration: co-immunoprecipitation, bimolecular fluorescence complementation, proximity ligation assay and surface plasmon resonance. The methods discussed herein allow for the study of PPIs in a wide variety of biological samples, including cell lysates, live cells, fixed cells and tissues, enabling the quantification of endogenous PPIs and exploration of the nature of PPIs. We also include a rudimentary framework for researchers to decide which experimental method best suits their research goals.

Key words: Co-immunoprecipitation, BiFC, SPR, PLA

The importance of protein-protein interactions (PPIs) and cellular migration

The central dogma dictates that the genome is transcribed into mRNA and subsequently translated into protein (Crick, 1970). James Watson himself, in a keynote address at the Miami Nature Biotechnology Winter Symposium in 2003, referred to DNA as "the script" and proteins as "the actors" in the complex play of life. Although reverse transcription, epigenetic regulation, prions, catalytic and non-coding RNA highlight novel conduits by which genetic information flows, the central dogma still remains one of the most enduring tenets of molecular biology (Mattick, 2003; Shapiro, 2009). Intrinsically, proteins are the crucial workhorses that facilitate the manifestation of a phenotypic end point. The presence, absence, mutation or modification of a particular protein will define the line between normal and pathophysiology. For example, in sickle-cell disease, a single amino acid substitution (glutamic acid to valine) at position 6 in the beta-subunit of the haemoglobin protein promotes haemoglobin aggregation under hypoxic conditions, which leads to the characteristic sickle-shaped erythrocytes and anaemia (Kassim and DeBaun, 2013). However, not every event in biology can be easily attributed to a single protein. A large majority of proteins do not function in isolation but rather require interaction with other proteins (homomeric or heteromeric) to exert an effect on cellular behaviour (Gandhi et al., 2006). The study of PPIs thus becomes

paramount to understanding the molecular underpinnings of health and disease. However, the detection and interpretation of these PPIs is challenging. The cell-type specific, time- and context-dependent expression of proteins and their binding partners, as well as their sub-cellular localisation, post-translational modifications and conformational changes before and after binding are all critical parameters that act in a combinatorial manner to allow for subtle fine-tuning of biological responses at all levels of physiology (Chautard et al., 2009; Zinzalla and Thurston, 2009).

The complexity of studying PPIs is further compounded by the individual nature of each PPI that occurs. PPIs are graded between the extremes of stable and transient interactions (Phizicky and Fields, 1995). Stable PPIs are those found among proteins belonging to a functional complex (e.g., haemoglobin, nucleoporins, integrin-ECM) and that form by default, i.e., the ideal complementary contacts among binding partners are intrinsically coded for by amino acid sequences, and thus, interactions occur with negligible extrinsic influences such as post-translational modifications. Correspondingly, stable PPIs are often retained over a longer time-course and are not easily disrupted. Such PPIs are less challenging to detect using conventional molecular techniques.

In contrast, transient PPIs typically require specific stimuli to encourage exposure of ideal complementary contacts before binding can occur. As implied, transient PPIs are not durable, because successful formation of the PPI results in a conformational change or interactions with other factors that undermine the precondition for binding (e.g. G-protein activation and deactivation, kinase phosphorylation and phosphatase dephosphorylation). Thus, transient PPIs play an important role as molecular switches that enable stringent and precise control of intracellular signal transduction. The temporary nature of such PPIs can pose tremendous difficulties in their detection. Nonetheless, a transient PPI can be detected with less difficulty in a particular cellular context if it occurs more frequently. Most biological events require the coordination of stable and transient PPIs for correct execution. Cellular migration, the focal biological event of our review, is no exception to this rule.

Nearly all cellular migrations involve an extracellular signal that is transduced internally by transient PPIs among members of a signalling cascade. These upstream initiators and effectors of migration are extremely diverse and remain the subjects of intense study and discovery. In contrast, downstream responses such as relinquishment of stable cell-ECM contacts and replacement by less stable (more transitory) cell-ECM contacts at the leading and withdrawing edges of the migrating cell appear more generic and are better understood (Palamidessi et al., 2013; Wehrle-Haller, 2012). Repeated iterations of these changes in PPIs eventually dictate when and where a cell should migrate to. During prenatal development, definition of the germ

layers of an early embryo already requires protein-directed cellular migration (Enders et al., 1978; Lawson and Pedersen, 1987; Poelmann, 1981). Rolling adhesion, subsequent extravasation and chemotactic movement of circulating immune cells to the site of infection within tissue also depends on appropriate PPIs between cells and the extracellular matrix (ECM) (O'Neill et al., 2000; Pribila and Shimizu, 2003; Yong and Khwaja, 1990). Wound repair and angiogenesis, which are key processes in the maintenance of organ integrity, can only occur correctly when keratinocytes and endothelial cells receive the appropriate signals for migration (Kim et al., 1994; Decline and Rousselle, 2001; Master et al., 2001). Such migration events during normal physiology also have parallels in pathology, and several diseases arise from or are exacerbated by deregulation of normal cell migration. Cancer, one of the most widely-diagnosed diseases of the 21st century that accounted for up to 13% of worldwide deaths in 2008 according to the World Health Organisation (WHO), sustains its nefarious growth by establishing its own network of blood vessels through de novo angiogenesis (Bergers and Benjamin, 2003; Chung et al., 2010). The most aggressive of cancers culminate their malignant progression in invasion and metastasis, which, is in essence a migratory incident (Hood and Cheresh, 2002; Roussos et al., 2011; Sethi and Kang, 2011).

It becomes apparent that PPIs and their associated signalling networks largely control migration in varying contexts. Precisely probing for the PPIs that occur during cellular migration can thus reveal pertinent therapeutic interventions to promote or prevent migration. Although many techniques have been developed to test for PPIs, herein we feature the most commonly and easily used experimental methods, old and new, that have contributed to and are currently expanding our understanding of these interactions in cell migration. We broadly categorise these methods into probe-based and direct-labelling techniques and evaluate each technique's advantages, limitations and future potential in elucidating PPIs that are pertinent to cell migration.

Methods for probing PPIs

Co-Immunoprecipitation (Co-IP)

Classically, the study of PPIs has been probe-based and driven by antibody-dependent approaches such as Western blotting, pull-down assays and a combination of these two methods, co-IP. These methods are contingent upon the availability of a specific recognition domain on an antibody that is raised against a particular epitope, which even allows for the specific capture of a protein based on its status (e.g., its activation state or presence of any post-translational modifications). Known as the gold standard in its field of assays, co-IP remains a popular method for the detection of PPIs. The idea of co-IP stems from using a specific antibody to purify and

detect antigens on a small-scale basis. The captured protein is immobilised onto a Protein A or G beaded support via the protein-specific antibody. From this stage, a co-IP procedure would then be able to pull-down any other protein-binding molecules from complex solutions through native interactions (see Figure 1). The power of co-IP comes from the modifiability of the assay to capture transient interactions that would otherwise be missed, or to map specific chains of events during the signalling cascade of interest.

The flexibility of co-IP was demonstrated by Master

et al in the characterisation of angiopoietin-1-dependent endothelial cell (EC) migration in angiogenesis (Master et al., 2001). Using antibodies that target phospho-proteins, in this case phospho-Dok-R, the authors demonstrated that the stimulation of EC migration by angiopoietin-1 is dependent on sequential PPIs between Dok-Nck-Pak that are involved in phosphorylation and activation of the indicated proteins. The versatility of co-IP was further extended to demonstrate that phosphorylation on a single tyrosine residue in Dok is necessary to activate this signalling axis to enhance EC

Fig. 1. Schematic depicting the underlying principle of co-immunoprecipitation (Co-IP). Cell lysates are incubated with bead-coupled antibodies specific to Protein A for immunoprecipitation. Protein B which physically interacts with Protein A remains bound while non-interacting proteins are washed off and the beads are pelleted by centrifugation. After elution, immunoblotting for Protein B is performed. A darker band appearing in the immunoblot for the migrating cells indicate that the protein interaction is enriched in cell migration.

migration. By mutating the tyrosine residue in Dok to remove the phosphorylation and activation of Dok, all sequential downstream PPIs were undetectable by co-IP, and the subsequent EC migration was impeded. This result highlights the ability of co-IP as a powerful tool to pinpoint PPIs to the resolution of a single amino acid. Co-IP is thus able to piece together crucial PPIs in a pair-wise manner to account for a migratory event through the use of specific antibodies that detect activated or other post-translationally modified members, within a protein complex. In addition, co-IP can highlight the domain architecture that is necessary for binding, whereas precise mutagenesis enables the detection of key single amino acid residues that anchor an entire protein complex. These versatile properties of a co-IP-based experiment can be particularly useful in peptide drug discovery which is aimed at mimicking native protein interactions that impact cellular migration.

Re-epithelialisation and migration of keratinocytes during wound healing is a complex process that requires the coordination of keratinocyte-ECM contacts and re-organisation of the cytoskeleton for directional motility. Sehgal et al used co-IP to highlight that integrin $\alpha 6 \beta 4$, once thought to be a minor player in laminin-332 patterning and deposition during keratinocyte migration, in fact plays a central role in enabling directional cell motility (Sehgal et al., 2006). Keratinocytes at the wound edge lay down the laminin-332 tracks on which they travel, and integrins $\alpha 3 \beta 1$ and $\alpha 6 \beta 4$ on keratinocyte plasma membranes are cognate binding partners of laminin-332 (Nguyen et al., 2000). Integrin $\alpha 3 \beta 1$ was first thought to be the major determinant of keratinocyte migration (deHart et al., 2003; Goldfinger et al., 1999). However, in the experimental context of dominant negative Rac-1 and Rac-1 inhibitors, a lack of Rac-1 activity caused circular laminin-332 deposition, which was an impediment to proper keratinocyte migration into the wound. Subsequently, a co-IP experiment clarified that integrin $\alpha 6 \beta 4$ is responsible for recruiting and activating Rac-1. In contrast, integrin $\alpha 3 \beta 1$ failed to co-precipitate with Rac-1, and Rac-1 recruitment by integrin $\alpha 6 \beta 4$ in turn activates cofilin to induce plectin/actin cytoskeletal rearrangements, which permitted integrin $\alpha 6 \beta 4$ to pattern laminin-332 deposition in a linear fashion for directional keratinocyte migration. In essence, co-IP has allowed the authors to link a less recognised integrin with Rac-1, and the latter's associated pathways, thus broadening the influence of integrin $\alpha 6 \beta 4$ in regulating the more subtle points of keratinocyte migration, for instance, the directionality of cellular movement.

Despite the utility and versatility of co-IP in (i) identifying PPIs, (ii) detailing the interfacing residues or unique characteristics of PPIs and (iii) associating PPIs with cellular migration in various contexts, co-IP does have limitations (Phizicky and Fields, 1995). The major limitation of co-IP is the inability to distinguish between direct PPIs or PPIs in a complex. For example, *in vivo* Protein A interacts with Protein B through protein C.

However, co-IP results will indicate that protein A interacts directly with B and would yield a false positive result (see Figure 1). Therefore, conclusions of direct PPI that are derived only from co-IP should be verified by another method that overcomes this limitation. Additionally, one crucial parameter is conspicuously missing from the co-IP experiments – the location within a cell where the PPI is occurring. This problem is inevitable because co-IP involves only proteins that are derived from cell lysates, therefore obviating any preservation of cellular structure and thus compartmentalisation of proteins. As such, non-physiological interactions may occur between proteins that are normally located in distinct cellular compartments. It is thus advisable that co-IP be performed in conjunction with immunofluorescence staining of the proteins involved in the PPI being probed to ascertain that the interacting proteins share the same intracellular compartment. In the Dok-R example, immunofluorescent staining followed by confocal imaging was performed to verify that Tek, Dok-R, Nck and Pak indeed co-localised and were found at the cell periphery of migrating ECs.

Given the myriad of proteins that are found in cell lysates, non-specific binding of unwanted proteins to the IP antibody is also possible. To minimize non-specific PPIs, one can enrich proteins in the pseudopodia protruding from the cell body compartment using polycarbonate microporous filters. Pseudopodia and cell bodies are then differentially scraped from the filter surface into lysis buffer for biochemical analysis. Using this method, it is possible to identify novel pseudopodia and cell body proteins, as well as study the spatiotemporal organization of signaling processes that regulate pseudopodium formation and cell polarity (Cho and Klemke, 2002; Tan et al., 2007; Wang and Klemke, 2007). In addition, thorough washing steps are typically involved to disrupt these weak interactions. Researchers can optimise the ionic strength of the buffers that are used in the assay, decrease the amount of target-specific antibody that is used to reduce signal noise, or pre-clear the samples by introducing non-specific antibodies to the cell lysate mixture prior to the actual IP steps.

Another consideration of a co-IP experiment is antibody contamination. The heavy and light chains of the antibody could be co-eluted with the complex of interest and will obscure the results on subsequent reducing SDS-PAGE runs. To circumvent this problem of antibody contamination, researchers may experiment with covalent immobilisation of the antibody to the beaded support. In this setup, beaded supports that are produced commercially can bind the primary amine groups on the antibodies, such that the antibody is permanently bound to the support. Another possibility for crosslinking the antibody to the Protein A/G-attached supports is through the use of crosslinkers, such as bisulfosuccinimidyl suberate (BS3) or its non-water soluble analogue, disuccinimidyl suberate (DSS). In this manner, covalent amide bonds are formed between the

antibodies and the beaded support. These methods aim to reduce the occurrence of antibody contamination during the analysis of results.

Finally, researchers may face the difficulty of ensuring a stable physiological environment for a PPI of interest to occur. This issue is perhaps most apparent when multicolour immunofluorescence staining identifies strict co-localisation of proposed interacting partners that cannot be proven to physically interact via co-IP. The PPI that is formed is likely weak or transient and unable to withstand the mechanical and chemical stresses that are exerted on it during the co-IP assay; this weakness severely limits the sensitivity of co-IP. The incorporation of a fixation or cross-linking step may be necessary prior to cell lysis to stabilise such PPIs. The stringency of wash steps after immunoprecipitation may also need to be reduced. These modifications to a typical co-IP protocol would require optimisation to ensure that false positives do not surface with the reduction of wash stringency, and that epitopes are not masked by fixation or cross-linking.

The continued reliance on these antibody-based

methods in many current laboratories is a testament to their reliability, and many of the newer techniques that have been developed to probe PPIs still leverage on antibody specificity. The proximity ligation assay (PLA) is a prime example of one such novel antibody-based technique and will be discussed in greater detail later in this review.

Bimolecular Fluorescence Complementation (BiFC)

BiFC is an *in vivo* direct-labelling technique based on the principle of fluorescence complex formation upon the interaction of two protein molecules (see Figure 2). Two putative interacting partners are individually fused to a fragment of a fluorescent protein. Upon interaction, the fluorescent protein complex reassembles and fluoresces (Kerppola, 2008; Kodama and Hu, 2012). Unlike FRET, BiFC does not involve radiative transfer of energy and therefore the obtained signal is stronger and offers a directly measurable readout. Because PPIs occur within live cells, the compartmentalisation of the interacting protein partners is retained, and the sub-

Fig. 2. Schematic depicting the underlying principle of bimolecular fluorescence complementation (BiFC). Cells must first be transfected to exogenously express both proteins of the prospective interaction as fusion proteins. Each protein is fused with either the N- or C-terminus of a split reporter which refolds into a functional fluorophore when the PPI of interest occurs. Appearance of fluorescence signals at the leading edge of a migrating cell confirms the subcellular localisation where PPIs are formed. BiFC can be coupled with FRET to identify higher order interactions involving three or more proteins. Refolding of the fluorophore when two proteins interact results in fluorescence emission that excites a second fluorophore fused to a third interacting partner. The emission of the BiFC fluorophore is quenched while the emission of the FRET fluorophore is enhanced.

cellular localisations of the PPIs can be resolved by confocal microscopy. Thus, if an interaction is detected by co-IP but not observed in a BiFC experiment, it could be an indication that co-IP has picked up a non-native PPI between partners that share distinct sub-cellular compartments in an intact cell. Conversely, the two proteins could interact, but in a different orientation that does not allow the two fragments of the fluorescent protein to assemble into a functional protein.

A wide variety of split-fluorescent reporters are now commercially available, including split-CFP, YFP, GFP and mDsRed, as well as their respective modified variants with superior performance (brighter fluorescence, higher signal-to-noise ratio) (Kodama and Hu, 2012). Using split-fluorescence reporters with non-overlapping emission spectra enables the simultaneous monitoring of multiple complex formations in the same cell. More recently, the repertoire of split-fluorescent reporters has been further expanded with the development of the near-infrared BiFC reporter iSPLIT, which was modelled after the PAS and GAF domains of the bacterial phytochrome RpBphP2 and improved upon by directed evolution (Filonov and Verkhusha, 2013). iSPLIT widens the spectral range and thus the number of split-fluorescence reporters that can be used simultaneously, and it further enables imaging of PPIs in live animals because its excitation and emission spectra lie beyond the wavelengths of the autofluorescent compounds found in animal tissues.

In the context of cell migration, BiFC demonstrated the importance of the integrin-linked kinase (ILK) and p38 β interaction in promoting serum-induced bladder cancer cell migration. Yu et al. demonstrated that ILK and p38 β elevation in invasive bladder cancer tissues as opposed to that of normal bladder urothelium (Yu et al., 2014). Interestingly, the specific inhibition of ILK activity institutes a selective loss of p38 β in bladder cancer cells. ILK phosphorylation of p38 β at specific residues was later shown to confer protection against the proteosomal degradation of p38 β . Live-cell imaging of bladder cancer cells transfected with p38 β and ILK that were fused with the N- and C-termini of Venus, respectively, indicated that ILK specifically interacts with p38 β in the cytoplasm. The advantage of using BiFC is twofold. First, an overexpression of the interacting partners tends to accentuate a phenotypic readout, which leads to the ability of BiFC to prove a successful PPI and the subsequent cellular phenotype. Second, in contrast with co-IP, BiFC provides an insight into the sub-cellular localisation of PPIs, and rules out interactions between proteins that do not naturally share an intracellular space or organelle because the assay requires the use of intact, living cells by default. In addition, when a truncated or loss-of-function ILK mutant was fused to the C-terminus of Venus, and subjected to a BiFC assay with p38 β , it was definitively shown that the kinase domain is crucial for the interaction with p38 β . Similar to co-IP, the option of truncation and mutagenesis of proteins that are involved

in a PPI of interest is absolutely viable to identify the protein domains responsible for the PPI. Depletion of either ILK or p38 β resulted in a substantial decrease in serum-directed chemotaxis of the bladder cancer cells, thus confirming a causal relationship.

Despite its utility in identifying PPIs *in vivo* using live cells with sub-cellular localisation resolution, BiFC does have its limitations. The often irreversible reassembly of the fluorescence reporter protein stabilises transient, native PPIs, making it possible to visualise them. However, the drawback to this is that the dynamics of interactions are lost because permanent reassembly of the split-fluorescent probe unnaturally prolongs the very interaction being studied. Furthermore, the absence of a BiFC signal does not necessarily mean that PPIs did not occur. Steric hindrances could lead to a non-optimal orientation of the two fragments of the fluorescent protein to assemble into a functional fluorescent protein, even though the proteins-of-interest have interacted. To work around this, a flexible linker region is sometimes incorporated into the fusion construct, thus allowing sufficient range of molecular motion to drive refolding of the fluorescent reporter (Kerppola, 2008).

BiFC also assumes that expression of the split-fluorescent reporter that is fused to the putative interacting partners does not interfere with the native structures of the proteins of interest. Alterations in native protein structure can cause residues required for stabilising a PPI to be obscured, which would result in a false negative readout. A fusion protein with abnormal structure may also expose residues to allow for the formation of non-native PPIs that out-compete the native interaction or cause non-native interactions to be interpreted as legitimate. A BiFC experiment should be complemented with immunocytochemistry and co-IP to definitively prove the localisation and existence of a PPI between two proteins of interest.

Another issue to consider in a BiFC experiment is that the extent of expression of the fusion proteins cannot be strictly controlled even when expressed from an inducible promoter. This is a problem that is inherent to any experiment in which overexpression from an exogenous construct is involved and is not unique to the BiFC method. Non-physiological levels of fusion protein expression could strain the cell transcription and translation machinery, which could lead to cell death or the induction of an abnormal cellular state. Alternatively, high non-endogenous expression of fusion proteins may result in fluorescent reporter refolding that is independent of PPI formation, giving a false positive result (Kerppola, 2008). As with any experiment, optimisation of the transfection efficiency or induction of fusion protein expression should be ensured.

The principles behind FRET and BiFC allow both methods to be used concurrently to further increase the number of PPIs that can be observed simultaneously. Most PPI detection methods only detect interactions between two proteins at a time. Yet, some PPIs occur in

ternary complexes and involve three or more proteins that are in physical contact with one another. An example of this situation is the formation of the pSmad2/3-TAP63-p53 complex in cholangiocarcinoma cells that promotes tumour cell migration, in addition to its survival (Lu et al., 2013). However, visualisation techniques for studying such ternary complex formations are lacking.

Recently, Shyu and colleagues established a BiFC-based FRET (BiFC-FRET) visualisation technique to study the formation of ternary complexes in living cells (Shyu et al., 2008). The BiFC-FRET technique encompasses the use of two mutant CFP and YFP proteins, known as Cerulean and Venus, respectively. In general, two proteins are conjugated to the two non-fluorescent fragments, as in the original BiFC technique. When an interaction successfully occurs, the two non-fluorescent fragments fuse to create the Venus fluorescent protein. In the presence of a third protein, which is fused to the Cerulean fluorescent protein, an interaction with the initially formed heterodimeric protein would bring Cerulean (FRET donor) into close proximity to Venus (FRET acceptor) and allow a successful FRET to occur. The visualisation of FRET in this case will be evident only upon the formation of a ternary complex. In their report, the team identified the formation of ternary complexes such as Jun-Fos-NFAT1 and Jun-Fos-p65 (Shyu et al., 2008).

An alternative method to detect and study ternary complex formation is known as the FRET-Fluorescence lifetime imaging microscopy (FLIM) assay (Kinoshita et al., 2007). This assay also relies on a FRET-based theory: two proteins that are part of a ternary complex are each conjugated to a chromophore. Changes in FRET signals in the presence of a third protein are then measured using the FLIM method. FLIM is designed to measure the fluorescence lifetimes of fluorescent proteins. When FLIM is applied as a downstream assay to FRET, protein-protein interactions and conformational changes of proteins in living cells can be spatially and temporally measured with high resolution. Whereas FRET occurrence is tracked by the decreasing lifetime of the donor chromophore in the presence of an acceptor chromophore, FRET-FLIM provides a certain advantage over traditional intensity-based FRET assays and has previously been discussed by Wallrabe and Periasamy (Wallrabe and Periasamy, 2005).

Because such techniques as BiFC-FRET and FRET-FLIM are still relatively novel, studies on ternary complex formations are still in their infancy stages. Future publications using such techniques will elucidate their role in cell migration.

Proximity Ligation Assay (PLA)

The need for a sensitive and specific assay to detect PPIs led to the development of the *in situ* PLA. PLA combines the robust and versatile properties of antibodies with a split-reporter assay to generate a

selective and specific method to detect PPIs. This method is similar to immunostaining (immunohisto- and immunofluorescence staining) in principle and allows for the detection of endogenous proteins down to single-cell resolution. This ability allows researchers to study PPIs in their most natural environment, within a cell, which is paramount in the context of cell migration.

In immunostaining, primary antibodies are used to target the proteins of interest, followed by the application of fluorophore-conjugated secondary antibodies to visualise the proteins of interest. This process provides information on the expression and co-localisation of the suspected interacting protein pairs. However, the limitation of microscopy resolution is a barrier to concluding whether the partners are indeed interacting or merely in close proximity. *In situ* PLA overcomes this limitation by replacing the secondary antibodies with a pair of proximity probes (antibodies conjugated to oligonucleotides) (see Figure 3). This pair of oligonucleotides acts as a template for the hybridisation and ligation of subsequently added connector oligonucleotides to form a circular DNA molecule. The hybridisation and ligation of the connector oligonucleotides is only possible if the proximal probes are close to each other (40 nm if employing secondary antibodies or 30 nm if primary antibodies are directly used), which underscores the most crucial innovation of the assay. The circular DNA acts as a template for infinite rolling circle amplification (RCA), a process performed by a DNA polymerase. This generates an extended single-stranded DNA molecule containing concatemeric copies of the original circular DNA sequence that remains attached to the proximal probes (Soderberg et al., 2006). These amplification products (AP) are then visualised using fluorescently labelled oligonucleotides that hybridise to a unique sequence on the AP. Because the AP contains numerous repeats of this sequence, the binding of the detection oligonucleotides generates a clear, localised fluorescence signal that is easily distinguishable from background readings. This feature greatly enhances the resolution of the assay. Because each PPI corresponds to one AP signal, the quantification of the number of interactions and the precise location of each interaction pair becomes possible, which provides further insights into cellular biology (Soderberg et al., 2006).

As mentioned in the BiFC section of this review, BiFC was used by Yu and colleagues to demonstrate the interaction between ILK and p38 β *in vivo* to substantiate its relevance to bladder cancer migration potential (Yu et al., 2014). Nonetheless, to validate their findings and ensure that endogenous ILK and p38 β indeed interacted in a serum-responsive manner, *in situ* PLA was their method of choice. In agreement with their BiFC observations, a sharp increase in the number of cytoplasmic PLA signals (each representative of an ILK-p38 β interaction) was observed when TSU-Pr1 cells were stimulated with 10% FBS. Treatment with the ILK inhibitor QLT-0267 prevented complex formation

between ILK and p38 β (as evidenced by the marked reduction of cytoplasmic PLA signals) and halved the serum-directed chemotaxis of serum-stimulated TSU-Pr1 cells (Yu et al., 2014).

In a study on wound-healing, PLA performed on primary human keratinocytes and mouse wound tissue sections also revealed the importance of ANGPTL4 in modulating interactions between keratinocytes and their ECM (Goh et al., 2010). ANGPTL4 is a secreted factor that is up-regulated by PPAR (Mandard et al., 2004) and hypoxia (Belanger et al., 2002) and has known implications in lipid and glucose metabolism (Oike et al., 2005) as well as breast cancer metastasis (Minn et al., 2005). It is cleaved post-translationally to give rise to N- and C-terminal fragments (nANGPTL4 and cANGPTL4) that are known to possess distinct functions. Goh and colleagues identified cANGPTL4-vitronectin and cANGPTL4-fibronectin interactions that occur in the wound bed and epithelium via PLA.

cANGPTL4 binding to vitronectin (or fibronectin) did not obviate cognate integrin $\alpha 5 \beta 1$ binding as visualised by triple PLA, wherein a PLA signal is only detected when all three members of the proposed tripartite complex interact. ANGPTL4 was subsequently found to stabilise vitronectin and fibronectin against matrix metalloprotease degradation, which in turn potentiates the activation of integrin-dependent intracellular signalling pathways that promote keratinocyte migration (Goh et al., 2010).

PLA offers researchers a convenient way to detect PPIs that occur between endogenous proteins of intact cells and tissue sections. This method preserves PPI localisation and ensures that only native interactions are highlighted, which positions PLA as a technique of high specificity. With the incorporation of an innovative amplification reaction that strengthens the signal output, PLA allows for detection sensitivity at the impressive resolution of a single PPI. PLA also allows for the

Fig. 3. Schematic depicting the underlying principle of the proximity ligation assay (PLA). Cells are fixed and permeabilised before incubation with antibodies specific to the proteins involved in the prospective PPI of interest. Secondary probes are added, consisting of secondary antibodies conjugated to oligonucleotide sequences. Subsequent ligation and rolling circle amplification (RCA) reactions are only permitted when the oligonucleotide sequences are within 40nm of each other. The amplification products (AP) of RCA contain a unique sequence to which fluorescent detection oligonucleotides bind to, producing a spot signal representative of individual PPIs of interest.

detection of transient PPIs in cells and tissues, up to sub-cellular levels because fixation of the cells is involved. This technique provides a relative but objective quantification of PPIs, which is a characteristic that is not readily achieved when using other techniques. However, PLA also has its own limitations. Being an antibody-based assay means that the success of PLA is still dependent upon ensuring ideal antibody binding conditions and is vulnerable to the stresses of wash steps which may compromise the sensitivity towards transient PPIs. Hence, this requirement emphasises the need for positive and negative controls for each PPI being tested. For example, when probing for an interaction between cANGPTL4 and vitronectin with PLA, Goh et al also probed for vitronectin and integrin $\beta 5$, which is a well-established cognate interaction (Goh et al., 2010). As a negative control, one can consider probing for proteins that do not share the same cellular compartment under physiological conditions (e.g., integrin $\beta 5$ and nucleoporin).

Another limitation is that PLA has a low upper limit on the number of interactions that can be simultaneously detected. The triple PLA experiment for the identification of the cANGPTL4-integrin $\alpha 5 \beta 1$ -vitronectin ternary complex most likely required careful optimisation of antibody binding conditions and of the time that was allowed for amplification and fluorescent-labelled oligonucleotide binding. Increasing the number of PPIs that are being simultaneously probed could be complex and liable to failure. Additionally, permeabilisation of cells to allow for access by the primary antibodies means that PLA is used as an end-point experiment and is not amenable to live-cell imaging.

Surface Plasmon Resonance (SPR)

The concept of SPR has previously been described (Helmerhorst et al., 2012). Briefly, surface plasmon is an electromagnetic wave that propagates along the surface of a thin metal layer. To achieve SPR, a beam of

polarised light is shone through a prism at a precise angle where it undergoes total internal reflection at the prism/metal/medium interphase and results in the excitation of the surface plasmon to generate the SPR. The angle of the light is extremely sensitive to changes in the reflective indexes of the medium that is adjacent to the metal layer; therefore, valuable statistics on protein interactions can be obtained by directly measuring the intensity of the reflected light. Experimentally, the protein of interest (ligand) is bound to a sensor chip that normally has a gold polymer interface. The suspected protein (analyte) solution is then allowed to flow over the sensor chip. When binding of the ligand and analyte occurs, the reflective index of the medium changes, and this change is dependent upon the molecular weight and number of proteins that are bound, among others. The characteristics of the individual interactions, whether they are transient or stable, strong or weak, fast or slow directly impact the changes in intensity of the reflected light that is measured and yields real-time and highly sensitive information on protein interaction kinetics and equilibrium constants.

One recent article demonstrated the use of SPR to analyse the binding of plasminogen (PIg) to its postulated receptor, thrombomodulin (TM), in promoting endothelial migration and invasion (Chen et al., 2013). This experiment was performed by measuring the binding kinetics of PIg and TM using a Biacore sensor, and the readout was given in terms of the association and dissociation rate constants. High values for the association and dissociation rate constants indicated that the PIg-TM binding and dissociation events occurred rapidly.

One potential drawback of the SPR technique is the possibility of the protein-of-interest losing its native configuration upon its attachment onto the sensor chip surface, or that its orientation on the chip may confer steric hindrance that could prevent analyte binding. To address this problem, researchers may have to consider alternatives, such as switching the ligand to become the

Table 1. Summary of methods for probing PPIs during cell migration.

		Co-IP	BIFC/FRET/FLIM	PLA	SPR
Experimental requirement	Probe-based	✓		✓	✓
	Direct labelling		✓		
	Intact cell (PPI localisation retained)		✓	✓	
	Cell lysate (PPI localisation lost)	✓			✓
	Live-cell imaging		✓		
Nature of PPIs	Stable	✓	✓	✓	✓
	Transient	✓*	✓	✓*	✓
	Post-translational modifications involved	✓		✓	✓
Readout	Semi-quantitative	✓	✓		
	Quantitative			✓	✓
	Affinity and kinetics				✓

*: denotes modification to protocol may be necessary.

analyte or optimising the attachment so that minimal binding heterogeneity to the sensor surface occurs. The presence of low-affinity or non-specific surface sites can also lead to an increase in non-specific binding effects at the surface of the sensor chip. Similarly, the pH and molarity of the buffers that are used will also influence the PPIs and questions remain as to whether these conditions reflect the endogenous context. Similar to co-IP, no subcellular localisation can be inferred from the interactions.

Despite these disadvantages, SPR has many advantages as well. In addition to it being a technique that is label-free, it provides rapid results and is highly sensitive; researchers are able to obtain real-time measurements of protein interactions and can determine the concentration of the analyte without the need for standard calibrations by applying calibration free concentration analysis (CFCA). CFCA provides an actual "active" concentration of the analyte that is compared to other techniques, such as ELISA or spectroscopic methods. This feature is critical, particularly in the pharmaceutical industries where the actual "active" concentration of an analyte is crucial to design an optimised production line. SPR is also the only technique that can provide data on the affinity constants (association and dissociation) of PPIs.

Conclusion

Cellular migration is a fundamental and important process that occurs during numerous biological activities, such as embryogenesis, angiogenesis, wound healing, inflammatory response and cancer metastases. Control of cellular migration is dependent upon PPIs that initiate and effect cytoskeletal remodelling alongside changes in cell-ECM contacts to enable a cell to move on its substratum in a directional manner. Given the multitude of proteins that coexist and interact within a cell at any point in time, it is often difficult to identify PPIs that are relevant to cellular migration. A reductionist approach to this problem is to demonstrate that a particular PPI associates strictly with a migratory event and that PPI formation is necessary and sufficient to promote cellular migration. To this end, probe-based and direct-labelling techniques have been developed to provide researchers a visual, semi- if not fully quantifiable readout of PPIs that drive the migratory events in cell biology. Each of these methods has its merits and shortcomings, but many serve complementary functions when applied in combination. Herein we have highlighted four methods, co-IP, BiFC (pure and hybrid versions), PLA and SPR which continue to enable researchers to gain insight into the molecular mechanisms that underlie cell migration. In summary, the decision to use one method over the other can be summarised through three main factors: (1) experimental requirement, (2) the nature of the PPIs being probed and (3) the preferred experimental readout

(see Table 1).

For example, if one chooses to employ co-IP for the identification of PPIs involved in migration, an antibody against proteins of interest must first be available, and the experimental setup, by default, forgoes the sub-cellular localisation of the PPI which could be important in some cases. Co-IP is more suitable for the detection of stable interactions; however, it is possible to stabilise transient interactions with the integration of a cross-linking step in its protocol. Although a researcher may choose to employ BiFC from the beginning because BiFC is directly capable of simultaneously capturing transient PPIs and the sub-cellular localisation where the PPI is occurring, the design of reporter-fusion protein constructs and optimisation of their *in vivo* expression may be costly and time-consuming. Hence, co-IP could be an efficient way for researchers to gain a foothold in demonstrating that the PPI indeed exists before committing to elucidating its localisation via BiFC.

Arguably, the best evidence for migration-related PPI formation is the observation of PPIs that form as migration occurs. Such observations can be made using BiFC and live-cell imaging in real time. Nonetheless, the readout of BiFC is only semi-quantitative because fluorescent labels only allow intensity-based inference of the frequency of PPI occurrence, and conclusions made from cells expressing exogenous, split-reporter-fused proteins may be viewed sceptically. These reservations can be overcome with PLA, which allows each PPI to be counted in non-migratory versus migrating cells which yields a precise quantitative result of whether a PPI is upregulated during cell migration. Moreover, probing is performed on endogenous proteins of unmodified cells, which alleviates any concerns over the non-native effects of the exogenous expression of split reporter-fused proteins.

As has been highlighted in this review, a greater depth into PPIs can be gained by truncating or mutating the proteins that are involved in a PPI of interest. Truncations or mutations that abolish PPI formation can reveal essential domains and residues that hold together entire protein complexes. Should further depth into the interaction be desired, SPR can be used to provide insight into the affinities and kinetics of binding.

To conclude, we have endeavoured to consolidate the advantages, limitations and considerations when using each of these methods to probe for PPIs in cell migration. The techniques discussed here are by no means exhaustive, but should suffice as a sound framework for researchers to decide on their preferred approach.

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Src is activated by the nuclear receptor peroxisome proliferator-activated receptor β/δ in ultraviolet radiation-induced skin cancer

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Abstract

Although non-melanoma skin cancer (NMSC) is the most common human cancer and its incidence continues to rise worldwide, the mechanisms underlying its development remain incompletely understood. Here, we unveil a cascade of events involving peroxisome proliferator-activated receptor (PPAR) β/δ and the oncogene *Src*, which promotes the development of ultraviolet (UV)-induced skin cancer in mice. UV-induced PPAR β/δ activity, which directly stimulated *Src* expression, increased *Src* kinase activity and enhanced the EGFR/Erk1/2 signalling pathway, resulting in increased epithelial-to-mesenchymal transition (EMT) marker expression. Consistent with these observations, PPAR β/δ -null mice developed fewer and smaller skin tumours, and a PPAR β/δ antagonist prevented UV-dependent *Src* stimulation. Furthermore, the expression of PPAR β/δ positively correlated with the expression of *SRC* and EMT markers in human skin squamous cell carcinoma (SCC), and critically, linear models applied to several human epithelial cancers revealed an interaction between PPAR β/δ and *SRC* and TGF β 1 transcriptional levels. Taken together, these observations motivate the future evaluation of PPAR β/δ modulators to attenuate the development of several epithelial cancers.

Keywords keratinocyte; PPAR beta/delta; Skin cancer; Src; UV

Subject Categories Cancer; Skin

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Introduction

The incidence of non-melanoma skin cancer (NMSC) is increasing at a high rate (3–8% yearly), with over one million estimated new cases each year in the United States alone (Madan *et al*, 2010). Squamous cell carcinoma (SCC) and basal cell carcinoma, which both derive from keratinocytes, are the most common types of NMSC. Although mortality due to NMSC is low, its morbidity is high and is accompanied by heavy personal burden and enormous, rising costs for society. NMSCs are associated with excessive and/or chronic exposure to ultraviolet (UV) radiation and mainly occur on sun-exposed areas. In particular, UVB radiation (290–320 nm, sunburn rays) predominantly affects keratinocytes because of its low skin penetration and is believed to act as a carcinogen (Ehrhart *et al*, 2003). Despite the prevalence of NMSCs, the mechanisms underlying their progression remain poorly defined.

Tumours have often been referred to as ‘wounds that never heal’. Gene expression profiling, which has revealed that a wound-response gene expression pattern predicts metastasis, survival likelihood and response to chemotherapy in cancer patients, supports this concept (Bissell & Radisky, 2001; Chang *et al.*, 2004; Farmer *et al.*, 2009; Nuyten & van de Vijver, 2006; Schafer & Werner, 2008). Apoptosis resistance, cell proliferation and migration, matrix remodelling, and angiogenesis are processes of life-saving importance after an injury but are also common features of tumour progression. The identification of molecular regulators of the mechanisms that acti-

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vate, sustain and eventually shut down these processes may advance our understanding of tumour development in general and of NMSC in particular.

Our previous work on skin wound healing in mice demonstrated the importance of the peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor superfamily, in tissue repair (Michalik *et al*, 2001). Injury strongly stimulates expression of PPAR α (NR1C1) and PPAR β/δ (NR1C2) in keratinocytes at the edges of skin wounds, and individual genetic deletion of these isotypes delays healing (Michalik *et al*, 2001). Both PPAR α and PPAR β/δ affect the early inflammatory reaction, but PPAR β/δ -null mice exhibit additional defects in healing resulting from impaired keratinocyte adhesion and migration, as well as increased apoptosis at wound edges (Di-Poi *et al*, 2002; Michalik *et al*, 2005; Tan *et al*, 2001; Tan *et al*, 2007). Thus, during wound healing, inflammation-induced PPAR β/δ controls processes that are also involved in carcinogenesis. Whether PPAR β/δ is pro- or anti-carcinogenic remains controversial, however, because both effects have been observed (Michalik *et al*, 2004; Muller-Brusselbach *et al*, 2007; Peters & Gonzalez, 2009; Schug *et al*, 2007; Schug *et al*, 2008). To address this question, we investigated PPAR β/δ function in keratinocyte-derived UV-induced skin tumours. Here, we unveil a previously unrecognized control by PPAR β/δ over a pro-oncogenic pathway, identifying PPAR β/δ as a key focus for further epithelial cancer studies.

Results

UV radiation stimulates PPAR β/δ expression and activity, enhancing skin tumour formation and progression *in vivo*

To test if PPAR β/δ is implicated in UV-dependent tumour development, we first investigated whether its expression responds to UV radiation. SKH-1 hairless mice were exposed to chronic low-dose UV radiation for up to 31 weeks to mimic repeated exposure to sunlight. Interestingly, PPAR β/δ expression increased in dorsal skin upon chronic UV exposure, PPAR α expression was reduced and PPAR γ expression was unchanged (Fig 1A, left) (supplementary

Fig S1). Additionally, the expression of *Tgfb1* and *Plin2*, two well-known PPAR β/δ target genes, was stimulated in wild-type but not in PPAR β/δ ^{-/-} mice, indicating activation of the receptor (Fig 1A). Importantly, when wild-type and PPAR β/δ ^{-/-} SKH-1 hairless mice were subjected to chronic UV radiation, tumour incidence and multiplicity were much higher in wild-type mice compared to PPAR β/δ ^{-/-} mice during the course of chronic UV exposure (Fig 1B). Wild-type mice fulfilled the criteria for withdrawal from the experiment (tumour larger than 9 mm in diameter) earlier than PPAR β/δ ^{-/-} mice (Fig 1C, D). Eventually, a 100% tumour incidence was reached in both genotypes after 22 weeks of UV exposure, which might have resulted from gene mutations arising from long-term UV exposure and associated modifications in gene expression in the PPAR β/δ ^{-/-} mice, which override the inhibitory effect of PPAR β/δ deficiency on tumourigenesis. Nevertheless, the growth rate of the tumours and their final size were higher in wild-type mice (Fig 1E).

To gain additional support for a PPAR β/δ -specific response to UV exposure, the SKH-1 mice were exposed to a single acute UV dose and sacrificed 24 h later. The UV treatment caused enhanced erythema in wild-type mice, characterized by an increased expression of inflammatory markers (supplementary Fig S2A). As with chronic irradiation, PPAR β/δ expression and activity were enhanced in the skin of wild-type animals upon acute UV exposure, especially in the epithelial compartment where carcinomas arise, as documented by the stimulation of two known target genes, *Tgfb1* and *Plin2* (Fig 1F) (supplementary Fig S2B). These observations suggest a major function for PPAR β/δ in the initiation and progression of skin tumours in response to UV irradiation.

Increased levels of activated PPAR β/δ enhance Src expression

Our previous gene expression analyses indicated a higher expression of the tyrosine kinase *Src*, a well-characterized proto-oncogene and enhancer of skin carcinoma (Guasch *et al*, 2007; Joseloff *et al*, 2002; Matsumoto *et al*, 2003; Matsumoto *et al*, 2004; Serrels *et al*, 2009; Yagi *et al*, 2007), in wild-type versus PPAR β/δ ^{-/-} primary keratinocytes (supplementary Fig S3A) (Montagner *et al*, 2011). In

Figure 1. PPAR β/δ is over-expressed and activated upon UV radiation and enhances skin tumour formation and growth.

- A Quantification of PPAR β/δ (left), *Tgfb1* (middle) and *Plin2* (right) expression by RT-PCR in non-tumoural dorsal skin of chronically irradiated (Ch-UV, +) or non-irradiated (−) PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice. Values from non-irradiated dorsal skin of PPAR β/δ ^{+/+} mice (left) or from irradiated dorsal skin of PPAR β/δ ^{-/-} mice (right) were set to 1. Means $\pm$ SEM of 13 PPAR β/δ ^{+/+} and 9 PPAR β/δ ^{-/-} mice are presented. *p*-values are **p* = 0.035 for PPAR β/δ (left); **p* = 0.025 and ***p* = 0.008 for *Tgfb1* (middle); ***p* = 0.078 and ****p* = 0.0003 for *Plin2* (right) calculated by two-tailed Student's *t*-test.
- B,C Percentage of PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice bearing tumours (B) and average number of tumours per mouse (C) measured at the indicated time points of UV exposure. For (C), *p*-values are **p* = 0.025, ***p* = 0.008, ****p* = 0.0001 calculated by two-tailed Student's *t*-test. Data for 13 PPAR β/δ ^{+/+} and 9 PPAR β/δ ^{-/-} mice are presented.
- D Percentage of PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice involved in the experiment over time. Mice were withdrawn from the experiment based on criteria given in the Material and Methods section.
- E Tumour growth rate (left) and final size (right) from PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice measured before sacrifice. Means $\pm$ SEM for 13 PPAR β/δ ^{+/+} and 9 PPAR β/δ ^{-/-} mice are presented. *p*-values are ***p* = 0.009 for tumour growth rate (left) and **p* = 0.045 for tumour size (right) calculated by two-tailed Student's *t*-test.
- F Measurement of PPAR β/δ (left), *Tgfb1* (middle) and *Plin2* (right) expression by RT-PCR in dermis and epidermis compartments of dorsal skin of PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice 24 h after acute UV (Ac-UV) irradiation. Values from dermis of non-irradiated dorsal skin of PPAR β/δ ^{+/+} mice (left) or from dermis of Ac-UV dorsal skin of PPAR β/δ ^{-/-} mice (middle and right) were set to 1. Means $\pm$ SEM for 12 PPAR β/δ ^{+/+} and 12 PPAR β/δ ^{-/-} mice are presented. Values are representative of three independent experiments; *p*-values are **p* = 0.025, ***p* = 0.007, ****p* = 0.002 for PPAR β/δ ; ****p* = 0.0003, **p* = 0.049, ***p* = 0.0093, ****p* = 0.0041, ****p* = 0.0004 from top to bottom for *Tgfb1*; ****p* = 0.0048, ****p* = 0.0013, ****p* = 0.0006, ****p* = 0.0009, ***p* = 0.0071, ****p* = 0.0035, ****p* = 0.0029 from top to bottom and from left to right for *Plin2* calculated by two-tailed Student's *t*-test; ns, not significant.

addition to Src, the Fyn and Yes tyrosine kinases, which also belong to the Src family kinases, were expressed in skin (Fig 2A) (supplementary Fig S3B). Interestingly, acute UV irradiation caused a

selective PPAR β/δ -dependent enhancement of Src mRNA and protein expression *in vivo* (Fig 2A, B) (supplementary Fig S3B). Similarly, Src mRNA and protein levels were consistently and strikingly

Figure 1.

higher *in vivo* in chronically irradiated wild-type skin versus *Ppar β/δ ^{-/-}* skin (Fig 2C, D). This effect was selective since the expression of *Fyn* and *Yes* was not affected after chronic irradiation (supplementary Fig S3C).

The highest PPAR β/δ -dependent Src expression occurred in the epidermis, mainly in keratinocytes of the lower layers (Fig 2A, E) (supplementary Fig S4) where PPAR β/δ has been localized (Michalik et al, 2001). Consistent with these observations, human immor-

Figure 2.

talized keratinocytes (HaCaT cells) stimulated with the PPAR β/δ agonist GW501516 exhibited higher Src mRNA and protein expression, similar to ANGPTL4, which was used as a positive control of PPAR β/δ activity (supplementary Fig S5A, B) (Kersten *et al*, 2000; Rieck *et al*, 2008). Characterization by 5' RACE and sequencing of the Src transcript induced in a PPAR β/δ -dependent manner by GW501516 in primary keratinocyte culture or by UV in mouse skin indicated its correspondence with the Src-001 transcript described in Ensembl (ENSMUST00000029175) (Fig 3A, B). *In silico* Src analysis revealed five peroxisome proliferator response elements (PPREs), which resemble the consensus PPRE motif, in the intragenic Src regulatory region (Fig 3A) (supplementary Fig S5C). These elements were potentially responsive to PPAR β/δ activation in a reporter assay (Fig 3C), indicating that Src is likely to be a PPAR β/δ target gene. This finding is in agreement with PPAR β/δ -induced SRC expression in the absence of *de novo* protein synthesis (supplementary Fig S5D).

To determine whether these PPREs serve as binding sites for PPAR β/δ , chromatin immunoprecipitation (ChIP) PPAR β/δ experiments followed by re-ChIP p300, a PPAR β/δ co-activator, were performed using lysates from control (wild-type) and PPAR β/δ -knockdown (KD) mouse keratinocytes (MKs, Fig 3D, E). The endogenous PPAR β/δ of KD cells was strongly downregulated by siRNA (supplementary Fig S5E). PPAR β/δ was constitutively associated with PPRE 3, 4 and 5 among the five PPREs identified *in silico* and tested in transactivation assays (Fig 3A, C), but p300 was recruited by PPAR β/δ only upon GW501516 activation. Thus, ligand activation appeared to be necessary for PPAR β/δ to regulate Src gene expression (Fig 3E), as for *Tgfb1* (supplementary Fig S6).

Collectively, these observations demonstrated that UV induced PPAR β/δ expression and activity. This activity in turn directly increased Src gene and protein expression, results that define Src as a novel PPAR β/δ target gene.

PPAR β/δ enhances the Src-dependent EGFR/MAPK Erk1/2 signalling pathway in response to UV

UV irradiation activates the EGFR/MAPK Erk1/2 pathway via Src (Bode & Dong, 2003; Fritsche *et al*, 2007), a key mechanism in skin photocarcinogenesis in mice and humans (Rho *et al*, 2011). The possibility that PPAR β/δ activates not only Src but also the entire Src/EGFR/Erk1/2 downstream pathway was tested next in various PPAR β/δ and Src gain- and loss-of-function models. Src and Erk1/2

phosphorylation upon UV treatment was higher in wild-type than in PPAR $\beta/\delta^{-/-}$ primary keratinocytes (supplementary Fig S7), confirming the importance of PPAR β/δ activity for UV-dependent full activation of Src/Erk1/2. KD of PPAR β/δ in HaCaT cells consistently abolished UV-induced activation of the EGFR/Erk1/2 pathway (Fig 4A) (supplementary Fig S8), as did the absence of PPAR β/δ in skin *in vivo* (Fig 4B, C). Conversely, activation of PPAR β/δ by its agonist GW501516 enhanced the UV-induced phosphorylation of EGFR (Tyr845) and Erk1/2 in HaCaT cells (Fig 4A, D). Importantly, this action of PPAR β/δ completely depended on Src activity because Src loss of function, either by pharmacological inhibition using a PP2-specific Src family kinase inhibitor or Src KD by siRNA (supplementary Fig S8), prevented an increase in EGFR and Erk1/2 phosphorylation (Fig 4A, D). Taken together, these observations provide evidence in support of a PPAR β/δ -dependent UV stimulation of the entire Src/EGFR/Erk1/2 signalling pathway. Finally, *Ets1*, whose expression is stimulated by activated Erk1/2 (Fitsialos *et al*, 2007), was enhanced in the UV-irradiated dorsal skin of wild-type mice only (Fig 4E).

Wild-type and *Ppar $\beta/\delta^{-/-}$* mice were then topically treated with the PPAR β/δ antagonist GSK0660 prior to acute UV irradiation. The UV-induced expression of two PPAR β/δ target genes, *Plin2* and *Tgfb1*, was prevented by GSK0660 (Fig 5A, B), which also abolished the increase in Src gene and protein expression (Fig 5C, D), resulting in both decreased phosphorylation levels of EGFR and Erk1/2 (Fig 5D) and stimulation of *Ets1* (Fig 5E). Interestingly, the drug did not affect expression of the pro-inflammatory markers *Ptgs2* (*Cox2*) and *Il6* (supplementary Fig S9A, B) or of *Ppar γ* and *Ppar α* (supplementary Fig S9C, D). However, UV-induced *Ppar β/δ* upregulation was abolished, suggesting a positive feedback loop in PPAR β/δ expression, possibly involving Src (Fig 5F). Taken together, these observations identified PPAR β/δ as a positive regulator of a major pathway in skin carcinogenesis via its effect on Src expression and activity.

PPAR β/δ coordinates a pro-tumoural gene program in advanced actinic keratosis

Skin tumour growth is associated with increased proliferation and migratory potential of keratinocytes, and in this process, Src promotes epithelial cell dedifferentiation during the epithelial-to-mesenchymal transition (EMT). The EMT might also be regulated by other

Figure 2. PPAR β/δ promotes Src expression in irradiated skin.

- Src, *Fyn* and *Yes* mRNA expression by RT-PCR in acutely (Ac-UV, +) and non-irradiated (–) dermis and epidermis of *Ppar $\beta/\delta^{+/+}$* and *Ppar $\beta/\delta^{-/-}$* mice. The value from irradiated dermis of *Ppar $\beta/\delta^{-/-}$* mice was set to 1. Means $\pm$ SEM are presented ($n = 6$ mice/genotype/group). Data are representative of three independent experiments; p -values are, from top to bottom and from left to right, $***p = 0.0001$, $***p = 0.0005$, $**p = 0.01$, $**p = 0.008$ for Src; $***p = 0.0008$, $*p = 0.017$, $***p = 0.0009$ for *Fyn*; $***p = 0.0004$, $***p = 0.0006$, $***p = 0.0005$, $***p = 0.0004$, $***p = 0.0008$, $***p = 0.0001$, $***p = 0.001$ for *Yes* calculated by two-tailed Student's t -test.
- Right, immunoblot of total Src in Ac-UV and non-irradiated skin of *Ppar $\beta/\delta^{+/+}$* and *Ppar $\beta/\delta^{-/-}$* mice. One of three independent experiments is shown ($n = 6$ mice/genotype/group). GAPDH was used as loading control; IB, immunoblot; left, quantification of the immunoblot (right) of total Src in Ac-UV and non-irradiated skin of *Ppar $\beta/\delta^{+/+}$* and *Ppar $\beta/\delta^{-/-}$* mice. Values are normalized to GAPDH, $n = 3$ mice/group; from left to right, $**p = 0.010$, 0.009 , t -test.
- Src mRNA levels in non-tumoural skin of chronically irradiated (Ch-UV) *Ppar $\beta/\delta^{+/+}$* mice and *Ppar $\beta/\delta^{-/-}$* mice. Value from irradiated skin of *Ppar $\beta/\delta^{-/-}$* was set to 1. Means $\pm$ SEM are given for 13 *Ppar $\beta/\delta^{+/+}$* and 9 *Ppar $\beta/\delta^{-/-}$* mice; $*p = 0.035$, t -test.
- Left, quantification of Src protein from the immunoblot (right) normalized to GAPDH ($n = 5$); $**p = 0.009$, t -test. Right, immunoblot of Src levels in Ch-UV non-tumourigenic skin of *Ppar $\beta/\delta^{+/+}$* and *Ppar $\beta/\delta^{-/-}$* mice ($n = 5$). One of three independent experiments is shown.
- Representative pictures of Src immunohistochemistry in Ch-UV skin of 13 *Ppar $\beta/\delta^{+/+}$* and 9 *Ppar $\beta/\delta^{-/-}$* mice. Scale bars, 50 μ m.

Figure 3.

direct or indirect PPAR β/δ -modulated signalling pathways such as the Tgf β 1/SMAD pathway, which could act on *Src* expression (Glick, 2012; Han *et al*, 2005; Hoot *et al*, 2008; Martins *et al*, 2009; Nakamura & Tokura, 2011; Newkirk *et al*, 2007). Thus, we examined the ability of PPAR β/δ to enhance this transition by measuring expression of EMT markers in actinic keratosis with moderate atypia (grade II) of wild-type and Ppar $\beta/\delta^{-/-}$ mice, including growth factors, transcription factors, cytoskeletal and cell surface markers, extracellular matrix proteins and regulators (Fig 6A). The expression of many of these markers was higher in wild-type mice than in Ppar $\beta/\delta^{-/-}$ mice, suggesting that the wild-type advanced grade actinic keratosis presented a higher risk for premalignant progression. Results of basement membrane analysis using laminin 332 (α 3 β 3 γ 2) immunofluorescence supported this hypothesis (supplementary Fig S10A). The basement membrane between the basal keratinocyte layer and the adjacent stroma exhibited a clear lining of laminin 332 in Ppar $\beta/\delta^{-/-}$ actinic keratosis with moderate atypia (grade II) whereas laminin 332 deposition was more diffuse and thicker in lesions of wild-type mice, reflecting perturbations of the basal layer. The proximity ligation assay (PLA) showed that these laminin 332 infiltrates in the basal and suprabasal epidermal layers in wild-type mice could bind β 4 integrin (Fig 6B, red positive signal quantified in Fig 6D) and transduce signal via Rac1 (Fig 6C, red positive signal quantified in Fig 6D). These observations were consistent with the increased expression compared to Ppar $\beta/\delta^{-/-}$ mice of EMT markers in wild-type grade II actinic keratoses, which displayed a higher level of proliferating keratinocytes and more intense Keratin 13 staining, an early marker of malignant progression susceptibility (supplementary Fig S10B and S11). Moreover, histological grading of SCCs from wild-type and Ppar $\beta/\delta^{-/-}$ mice according to the Broders' classification (Broders, 1921) showed that wild-type mice developed more advanced SCCs (Fig 6E) (supplementary Fig S12). However, because of the conditions imposed by the Veterinary Office (see Material and Methods for details), it was not possible to obtain more advanced UV-induced skin tumours (Grade IV according to Broders' classification) to observe metastatic SCCs. Interestingly, EMT markers were also expressed at higher levels in grade II SCCs from wild-type compared to Ppar $\beta/\delta^{-/-}$ mice at the mRNA and protein levels. N-Cadherin staining confirmed this result (supplementary Fig S13).

Collectively, these observations suggested that keratinocytes of wild-type mice were more likely to invade the surrounding stroma. This characteristic represents a higher risk of malignant conversion in these mice compared to the Ppar $\beta/\delta^{-/-}$ mice.

Correlation between PPAR β/δ and Src expression in various human carcinomas

We wished to investigate whether the proto-oncogene *Src* is also a PPAR β/δ target in human SCC. Interestingly, the levels of *SRC* and *PPARB/D* expression were highly and significantly correlated in these tumours (Fig 7A), as were the expression levels of *PPARB/D* and *TGFB1* and *MMP19*, respectively, and to a lesser extent, *MMP2*, *VEGFA*, *VIM* and *SNAIL* (Fig 7B, C) (supplementary Fig S14A–D). These data suggested that, as in UV-irradiated mouse skin, PPAR β/δ regulates *SRC* expression in human SCC, which is correlated with the expression of EMT markers.

To further examine the underpinnings of these observations, we investigated the relationships among *SRC*, *TGFB1* and *PPARB/D* expression levels in human carcinomas from various organs. More specifically, we tested whether the degree of linear dependency between *SRC* and *TGFB1* mRNA levels (degree of correlation) is influenced by the abundance of *PPARB/D* mRNA present in the tumour (the significance of the interaction term *TGFB1*: *PPARB/D* of the linear model $SRC \sim TGFB1 + PPARB/D + TGFB1: PPARB/D$). This linear model was separately fitted to nine human tumour types, including skin SCC (Fig 7D–F) (supplementary Fig S14E–I). The least square estimation of the model parameter revealed that the *TGFB1*: *PPARB/D* interaction coefficient term was significantly different from zero for lung (0.17 [0.06–0.29], 95% confidence interval in square brackets) and ovarian (0.15 [0.04–0.27]) carcinomas and oral squamous cell carcinoma (OSCC) (0.009 [0.003–0.014]). Combining these nine independent interaction coefficients into a random effects model, a method commonly used in statistical meta-analysis, produced a significant summarized interaction coefficient (0.081 [0.014–0.146]; Fig 7F). Results for SCC show that patients with a *PPARB/D* mRNA abundance lower than the median value had an absence of linear dependency between *SRC* and *TGFB1* ($p = 0.75$; Fig 7D) but that those with a *PPARB/D* abundance above or equal to the median of the dataset showed a significant linear relationship ($p = 0.0336$; Fig 7E). Results with the ovarian carcinoma

Figure 3. *Src* is a direct PPAR β/δ target gene.

- Schematic structure of the murine *Src* gene. Untranslated and translated exons of *Src*-001 transcript and position of primers used for 5' RACE are depicted as indicated. GSP, gene-specific primer.
- Identification of the *Src* transcript isoform expressed in wild-type primary keratinocyte cultures treated with GW501516 (100 nM) for 24 h (lane 1) and total skin of SKH-1 wild-type mice after acute irradiation (lane 2). In both samples, a major 650-bp product was obtained. Sequencing of the isolated DNA bands indicated that the transcripts corresponded to the *Src*-001 transcript (ENSMUST00000029175).
- PPAR β/δ activity on wild-type or mutated PPRES in the *Src* promoter (supplementary Fig S5) via luciferase assays in NIH3T3 cells treated with GW501516 (300 nM) or vehicle (DMSO). Fold inductions were calculated as the ratio of firefly luciferase to Renilla luciferase activity using DMSO-treated NIH3T3 cells transfected with a TK-3PPRE construct designated as 1. Values are shown as the mean $\pm$ SD ($n = 3$). p -values are, from left to right, *** $p = 0.0002$, 0.0003, 0.0001, 0.0032, 0.0001, 0.0008 calculated by two-tailed Student's t -test. One of three independent experiments is shown.
- Representative results of ChIP experiments using anti-PPAR β/δ antibody (D) followed by re-ChIP with anti-p300 (E) performed in mouse keratinocytes (MKs) downregulated for PPAR β/δ expression by siRNA [knockdown cells (KD)] or not (wild-type cells, WT) treated or not with GW501516. The results show a PCR amplification of the PPRES, 4 and 5 sites. Preimmune serum (p.i.) served as a control for ChIP. Primer sequences are given in supplementary Table S3. Data are representative of $n = 3$ independent experiments.

Figure 4. PPAR β/δ -dependent upregulation of Src expression enhances EGFR/Erk1/2 signalling upon UV exposure *in vitro* and *in vivo*.

- A** Immunoblots of Src, pTyr845 EGFR, total EGFR, pErk1/2 and total Erk1/2 levels from whole-cell lysates of HaCaT cells transiently transfected with control (Ctrl), *Ppar β/δ* or Src siRNA, treated 24 h with GW501516 or DMSO, and then subjected to UVB radiation (40 mJ/cm²) before harvesting 30 min later. GAPDH was used as a loading control. Data are representative of three independent experiments.
- B,C** Immunoblot of pTyr845 EGFR, total EGFR, pErk1/2 and total Erk1/2 levels in protein extracts from chronically irradiated (Ch-UV) (B) or acutely irradiated (Ac-UV) (C) dorsal skin of *Ppar $\beta/\delta^{+/+}$* and *Ppar $\beta/\delta^{-/-}$* mice. Data are representative of three independent experiments.
- D** Immunoblot of pTyr845 EGFR, total EGFR, pErk1/2 and total Erk1/2 levels from whole-cell lysates of HaCaT cells treated with the PPAR β/δ agonist GW501516 or DMSO for 24 h before UVB exposure (40 mJ/cm²) in the presence or absence of a Src family kinase inhibitor (PP2), and harvested 30 min later. Data are representative of three independent experiments.
- E** Real-time RT-PCR of *Ets1* mRNA expression in non-irradiated and Ac-UV dorsal skin of *Ppar $\beta/\delta^{+/+}$* and *Ppar $\beta/\delta^{-/-}$* mice. Data are representative of three independent experiments for 12 *Ppar $\beta/\delta^{+/+}$* and 12 *Ppar $\beta/\delta^{-/-}$* mice; *p*-values are **p* = 0.032, ***p* = 0.009 calculated by two-tailed Student's *t*-test.

Figure 5. Pharmacological inhibition of PPAR β/δ prevents UV-induced Src expression and Src-dependent activation of EGFR/Erk1/2 signalling *in vivo*. Dorsal skin of *Pparβ/δ*^{+/+} and *Pparβ/δ*^{-/-} mice was topically treated with the PPAR β/δ antagonist GSK0660 or vehicle (Veh; 70% ethanol) prior to acute UV irradiation (Ac-UV); mice were sacrificed 24 h later.

A–C Quantification of *Plin2* (A), *Tgfb1* (B) and *Src* (C) mRNA expression via real-time RT-PCR. Means $\pm$ SEM are presented ($n = 6$ mice/genotype/group). Data are representative of two independent experiments; p -values are * $p = 0.0190$, ** $p = 0.0066$, *** $p = 0.0004$ for *Plin2*; * $p = 0.0421$, 0.0153 (from left to right) and ** $p = 0.0098$ for *Tgfb1*; * $p = 0.0428$, ** $p = 0.0098$, *** $p = 0.0045$ for *Src* calculated by two-tailed Student's t -test; ns, not significant.

D Immunoblot of total Src, EGFR, p-Tyr845 EGFR, Erk1/2 and p-Erk1/2 protein in extracts from dorsal skin of *Pparβ/δ*^{+/+} and *Pparβ/δ*^{-/-} mice. Data are representative of two independent experiments ($n = 12$ mice/genotype/experiment).

E Quantification of *Ets1* mRNA expression by real-time RT-PCR. Means $\pm$ SEM are given ($n = 6$); p -values are * $p = 0.0324$ and ** $p = 0.0091$, 0.082 (from left to right) calculated by two-tailed Student's t -test.

F Quantification of *Pparβ/δ* expression via real-time RT-PCR. Means $\pm$ SEM are presented ($n = 6$ mice/genotype/group). Data are representative of two independent experiments; p -values are ** $p = 0.0083$ and *** $p = 0.0006$ calculated by two-tailed Student's t -test; ns, not significant.

Figure 6.

and OSCC datasets led to similar inferences (supplementary Fig S14F–I).

Thus, our statistical modelling provided evidence that the expression level of *PPARB/D* influences the degree of correlation between *SRC* and *TGFB1* levels across a wide variety of human carcinomas, an observation that supports our hypothesis of a direct influence of PPAR β/δ on *SRC* and *TGFB1* transcription. Taken together, our observations and analyses indicate that PPAR β/δ regulates *SRC* in human SCC, notably in skin SCC, and that this regulation also occurs in carcinomas of various tissue origins, thus revealing a broad pro-tumorigenic potential of PPAR β/δ via *SRC* upregulation.

Discussion

Our study demonstrates that PPAR β/δ promotes skin cancer by enhancing Src activity in response to UV radiation. UV is a skin carcinogen causing both DNA damage resulting in tumour initiation and activation of signalling cascades that promote tumour development (Devary *et al*, 1993; Matsumura & Ananthaswamy, 2004; Rho *et al*, 2011). The signalling pathway involving the tyrosine kinase Src, the tyrosine kinase receptor EGFR and the MAPK Erk1/2 cascade is activated during UV-induced skin inflammation and carcinogenesis, promoting initiation, progression and malignant conversion. This pathway is therefore a target for epithelial cancer prevention (Fritsche *et al*, 2007; Rho *et al*, 2011). Importantly, this pathway and its cellular effects may also be modulated by other PPAR β/δ -dependent or -independent signalling pathways, such as Tgf β 1/SMAD.

To date, the nuclear receptor PPAR β/δ has been less studied than PPAR α and PPAR γ , which are drug targets for the treatment of dyslipidemia and type 2 diabetes, respectively. Recently, the US Food and Drug Administration released safety information linking pioglitazone, which targets PPAR γ , to increased risk for bladder cancer. Although there is growing interest in developing PPAR β/δ agonists to treat dyslipidemia, no PPAR β/δ compound has been brought to market, and the role of this isotype in tumorigenesis has been debated (Michalik *et al*, 2004; Peters & Gonzalez, 2009; Peters *et al*, 2012).

Our study has yielded the first insights into how PPAR β/δ promotes UV-induced skin tumourigenesis (Fig 7G). *In vivo* UV

exposure increases PPAR β/δ expression at the transcriptional level and its activity. Once activated, PPAR β/δ upregulates Src expression and activity, which in turn, boosts EGFR/Erk1/2 signalling. Thus, in our mouse model, PPAR β/δ sensitizes keratinocytes to UV, promotes actinic keratosis development and progression, and stimulates the expression of EMT markers that affect basement membrane integrity. Importantly, *PPARB/D* expression and *SRC*, *MMP19* and *SNAIL* expression directly correlate in human skin SCC, which may indicate a direct effect of PPAR β/δ on EMT initiation. Furthermore, our meta-analysis of PPAR β/δ activity in various epithelial tumours, including human skin SCC, revealed a positive interaction between *PPARB/D* expression levels and those of two of its targets, *SRC* and *TGFB1*. Interestingly, the identification of this relationship in large independent datasets of various tumour types, particularly in lung, colon and ovarian cancers known for their high levels of Src expression and activity, strongly supports a regulatory role for PPAR β/δ in human carcinomas. We speculate that early in skin actinic keratosis formation, PPAR β/δ regulates Src and may act, in concert with other genetic change, to promote tumour progression by increasing keratinocyte migratory and proliferative properties. Our data help to define the pro-carcinogenic properties of PPAR β/δ in skin carcinoma and most likely in carcinomas from other tissue origins, as well, and identify *Src* as a direct PPAR β/δ target gene, at least in mouse.

In accordance with our observations, ErbB2 activation, in concert with ErbB3 and ErbB4, leads to the expression of *FABP5*, an intracellular lipid binding protein, which plays a crucial role in PPAR β/δ transcriptional activation in the MCF7 cell line, suggesting that the EGFR superfamily could also act upstream of PPAR β/δ to regulate its activity in a model of breast cancer (Kannan-Thulasiraman *et al*, 2010). Although its consequences remain unknown, the expression of PPAR β/δ has been reported to be increased in skin pathologies such as the lesional skin of psoriatic patients (Romanowska *et al*, 2010; Westergaard *et al*, 2003) and in human skin biopsies of pre-malignant and malignant skin carcinoma (Nijsten *et al*, 2005; Sertznig *et al*, 2008). In the latter, increased PPAR β/δ expression has been associated with a significant increase in microvessel density and strongly correlates with the expression of COX2, an enzyme implicated in skin carcinoma development (Trifan & Hla, 2003). Interestingly, our previous findings of PPAR β/δ participation in keratinocyte survival, proliferation and migration during skin wound healing (Tan *et al*, 2007) are somewhat analogous to the

Figure 6. PPAR β/δ upregulates pro-tumoural marker gene expression and laminin 332 deposition in actinic keratosis, enhancing SCC progression.

- mRNA expression of epithelial-to-mesenchymal transition (EMT) markers in 20 actinic keratoses with moderate atypia (grade II) of *Ppar β/δ ^{+/+}* and *Ppar β/δ ^{-/-}* mice by real-time RT-PCR. Means $\pm$ SEM are presented; *p*-values are ***p* = 0.0077 (*Tgf β 1*), ****p* = 0.0009 (*Krt13*), **p* = 0.046 (*Vim*), **p* = 0.045 (*Snai1*), **p* = 0.032 (*Snai2*), **p* = 0.028 (*Gsc*), ***p* = 0.009 (*Itga6*), **p* = 0.025 (*Itga6*), **p* = 0.022 (*Itgb1*), **p* = 0.013 (*Itgb6*), **p* = 0.035 (*Cad12*), **p* = 0.020 (*Col7a1*), **p* = 0.035 (*Mmp19*), **p* = 0.025 (*Lam α 3*), **p* = 0.043 (*Mmp2*) calculated by two-tailed Student's *t*-test. The full gene names are given in supplementary Table S2.
- Representative immunofluorescence staining of PLA experiments for laminin 332/ β 4 integrin (red spots) in actinic keratosis with moderate atypia (grade II) sections from *Ppar β/δ ^{+/+}* and *Ppar β/δ ^{-/-}* mice. DAPI, blue. Scale bars, 50 μ m.
- Representative immunofluorescence staining of PLA experiments for β 4 integrin/Rac1 (red spots) and laminin 332 in actinic keratosis with moderate atypia (grade II) sections from *Ppar β/δ ^{+/+}* and *Ppar β/δ ^{-/-}* mice. DAPI, blue. Scale bars, 50 μ m.
- Quantification of PLA staining. Average number of PLA signals (red spots) per nucleus from *n* = 10 actinic keratoses with moderate atypia sections/genotype (**p* = 0.024, *t*-test).
- Actinic keratosis (AK) with grading of cellular atypia (mild, grade I; moderate, grade II; severe, grade III) and SCC distribution in 25 tumours collected from each *Ppar β/δ ^{+/+}* and *Ppar β/δ ^{-/-}* mouse graded histologically in a blinded manner according to Rowert-Huber *et al* and to the Broders' classification, respectively.

Figure 7.

tumour-development functions described in the current investigation, providing a further example of the molecular parallels between wound repair and cancer. It is intriguing to note that we and others have also described PPAR β/δ as a pro-differentiating factor in keratinocytes (Burdick *et al*, 2007; Peters & Gonzalez, 2009; Tan *et al*, 2001). Similarly, a pro-differentiation function has been attributed to the MAPK-Erk1/2 pathway in keratinocytes, a pathway that is clearly pro-carcinogenic in skin tumours (Rho *et al*, 2011; Seo *et al*, 2004). These apparently contradictory pro-differentiation and pro-carcinogenic roles may be due to different environmental stimuli and/or genetic contexts, which could lead to different kinetics and amplitude of Erk1/2 stimulation, switching the spectrum of their target proteins. This model could also accommodate a role for PPAR β/δ . Indeed, increasing evidence suggests that physiological or pathological contexts, including the genetic background, drive PPAR β/δ functions and might influence the extracellular signals, endogenous ligands produced, ligand-binding protein expression and post-translational modifications. These parameters are likely to affect the level and activity of PPAR β/δ and the expression pattern of its numerous target genes.

This hypothesized complexity could explain the between-study discrepancies regarding the role of PPAR β/δ in skin carcinogenesis. For example, Kim *et al* explored the anti-cancer properties of PPAR β/δ in skin using DMBA/TPA-induced carcinogenesis, a context in which tumour development was more severe in PPAR $\beta/\delta^{-/-}$ mice than in wild-type animals, suggesting that PPAR β/δ attenuates the development of chemically induced skin cancer (Kim *et al*, 2004). Inhibition of keratinocyte proliferation was thought to mediate this effect of PPAR β/δ mainly via the activity of protein kinase C α . However, in transgenic mice that over-express protein kinase C α , the susceptibility to DMBA/TPA-induced skin tumourigenesis was not affected (Cataisson *et al*, 2003; Wang & Smart, 1999). Moreover, induction of skin cancer by DMBA/TPA involves different molecular mechanisms from those associated with human skin cancer attributable to chronic UV exposure (Boukamp, 2005; Matsumura & Ananthaswamy, 2002). Notably, tumour initiation with a unique topical sub-carcinogenic dose of a genotoxic carcinogen (DMBA) primarily induces *Ha-Ras*-activating mutations, whose frequency reaches only 10–20% in human SCCs and basal cell carcinomas, reflecting a marginal contribution of this oncogene to these

carcinomas (Campbell *et al*, 1993; Lieu *et al*, 1991; Pierceall *et al*, 1991). In this context, our proposed model pathway could be masked by such *Ha-Ras*-activating mutations, rendering the MAPK Erk1/2 activation upstream independent of Src/EGFR stimulation.

Moreover, this apparent controversial function of PPAR β/δ in cancer development is also illustrated in other cancer types for which several studies have suggested an attenuation of carcinogenesis by PPAR β/δ , while others support a tumour-promoting role (reviewed in Michalik *et al*, 2004; Peters & Gonzalez, 2009; Peters *et al*, 2012). For instance, studies indicate that colon carcinogenesis is exacerbated in the absence of PPAR β/δ expression and/or that ligand activation of PPAR β/δ attenuates tumourigenesis (Harman *et al*, 2004; Hollingshead *et al*, 2008; Reed *et al*, 2004). In contrast, other studies have shown that genetic disruption of PPAR β/δ inhibits colorectal tumourigenesis and that ligand activation of PPAR β/δ promotes tumourigenesis (Gupta *et al*, 2004; Park *et al*, 2001; Wang *et al*, 2006; Zuo *et al*, 2009). Many of these studies are based on PPAR β/δ mRNA or protein expression levels, which do not necessarily reflect the ligand-dependent activity of the receptor. Interestingly, the expression of both PPAR β/δ and COX-2 in tissues of colorectal cancer patients leads to liver metastases, a condition associated with poor prognosis (Yoshinaga *et al*, 2011). PPAR β/δ gene expression is upregulated in patients with non-small cell lung cancer and correlated with the expression of *VEGFA* and *COX2*. Moreover, cell lines with relatively high PPAR β/δ activity (e.g. H441 and H358) exhibit proliferative and pro-survival responses, which are absent or minimal in cells with low levels of the receptor (e.g. A549) (Genini *et al*, 2012). In addition, PPAR β/δ , via its regulation of *ANGPTL4* gene expression, is suggested to be implicated in MDA-MB-231 cancer cell invasion into a three-dimensional matrix (Adhikary *et al*, 2012). These findings suggest a tumour-promoting function for PPAR β/δ , in accordance with our data on skin carcinoma, and this diversity of PPAR β/δ actions underscores the need for more work in mouse models and human tissue samples to decipher the complex PPAR β/δ functions in tumourigenesis. An illustration of this complexity is the importance of the isotype and expression levels of fatty acid-binding proteins in directing the same ligand towards RAR or PPAR β/δ . The latter promotes the pro-carcinogenic function of PPAR β/δ in breast tumour development (Kannan-Thulasiraman *et al*, 2010; Schug *et al*, 2007; Schug *et al*, 2008).

Figure 7. PPARB/D and SRC mRNA expression are correlated in human skin SCC and in other types of human carcinomas.

- A–C Correlation between PPARB/D and SRC (A), PPARB/D and TGF β 1 (B) and PPARB/D and MMP19 (C) expression as assessed via real-time RT-PCR of RNA extracted from human SCC biopsies ($n = 9$). p -values are calculated by two-tailed Student's t -test.
- D,E Interaction between TGF β 1 and PPARB/D underlies SRC expression levels. All 42 SCCs were sorted by PPARB/D expression. Samples with either the highest ($n = 21$; top 50% of the samples) or lowest ($n = 21$, bottom 50% of the samples) PPARB/D levels were identified. (D) Least squares regression between TGF β 1 and SRC expression levels in samples with the lowest PPARB/D expression. The black line represents the least square fit. The observed linear regression coefficient (-0.23) was not significantly different from zero (Student p -value = 0.75). (E) Linear regression between TGF β 1 and SRC levels in the 21 tumour samples with the highest PPARB/D expression revealed a significant linear relationship.
- F Table presenting the interaction coefficient β_3 (estimate) and its 95% confidence interval for the linear model $SRC \sim \beta_0 + \beta_1TGF\beta1SRC + \beta_2PPAR\beta/D + \beta_3TGF\beta1SRC:PPAR\beta/D$. The summarized meta-analysis of the interaction coefficient was estimated using a random effects model.
- G Model of the molecular function of PPAR β/δ in UV-induced skin tumours. UV irradiation induces PPAR β/δ gene expression and activation. Once activated, PPAR β/δ drives the expression of *Src*, which correlates with an increased *Src* protein level and higher kinase activity. This activity leads to the activation of the *Src*-dependent EGFR/Erk1/2 signalling pathway, which drives the expression of genes involved in the epithelial-to-mesenchymal transition (EMT). In coordination with other PPAR β/δ -dependent or -independent mechanisms and/or genetic defects, this mechanism enhances skin tumour formation and progression upon UV exposure, identifying PPAR β/δ as a putative inducer of carcinoma.

Src-centred investigations have thus far concentrated on the mechanisms of post-translational regulation of its kinase activity, but little is known regarding the factors regulating the expression of its gene. The observation that PPAR β/δ stimulates *Src* expression sheds new light on this powerful oncogene, and interest is growing in *Src* as a potential therapeutic target in skin carcinoma treatment (Serrels *et al*, 2009). Our study also raises fundamental biological questions related to cancer therapy. Inhibitors of *Src* are used in the treatment of many carcinomas but are associated with side effects (Kopetz *et al*, 2007). Because *Src* is a target of PPAR β/δ , specific antagonists of the receptor might be considered for the treatment of such cancers and/or prevention of malignant progression of actinic keratosis. Given that PPAR β/δ is a promising molecular target in the prevention and treatment of metabolic diseases such as dyslipidemia, obesity and diabetes, we suggest a careful evaluation of the use of high-affinity PPAR β/δ agonists such as GW501516, which is currently in phase IV clinical trials for the treatment of dyslipidemia (Ooi *et al*, 2011).

Materials and Methods

Animal model and UVB irradiation

SKH-1 hairless mice (Charles River) were crossed with SV129/BL6/J Ppar β/δ wild-type or Ppar $\beta/\delta^{-/-}$ mice to obtain SKH-1/Ppar β/δ wild-type and SKH1/Ppar $\beta/\delta^{-/-}$ mouse strains. Mice were housed in quarters with a 12/12-h light/dark cycle and maintained with water and food *ad libitum*. For acute and chronic UV exposure, mice were UV irradiated on their backs with a GL40E 40W tube (SNEE), which emits most of its energy within the UVB range (90%; emission spectrum 280–370; 10% UVA). Doses of UVB (312 nm) and UVA (370 nm) were monitored using an appropriate radiometer.

For acute exposure, female SKH-1 wild-type and Ppar $\beta/\delta^{-/-}$ mice ages 10–12 weeks were irradiated on their backs with the UV lamp. UVB radiation emission was controlled using a radiometer until a dose of 120 mJ/cm² was delivered. Non-irradiated age-matched mice were used as controls. Twenty-four hours after UV irradiation, animals were sacrificed using CO₂ gas, following by the acquisition of dorsal skin samples that were directly frozen in liquid nitrogen or prepared for histological analysis. For GSK0660 treatment, 200 μ l of GSK0660 (625 μ g/ μ l in 70% ethanol; Sigma, G5797) was applied topically on the back 1 h prior to UV exposure.

For chronic treatment, animals were irradiated on their backs three times per week with 70 mJ/cm² of UVB, which was monitored using a radiometer. The time of tumour appearance, number and size were monitored twice per week. Mice with one tumour reaching 9 mm in diameter were sacrificed using CO₂ gas in accordance with the requirements of the Veterinary Office of the Canton Vaud (Switzerland) and Federal Swiss Veterinary Office Guidelines. Non-irradiated aged-matched mice were simultaneously used as controls and handled in the same fashion as the irradiated animals. Dorsal tumours or non-tumoural irradiated skin samples were then obtained and prepared as described above.

Tumour grading

Mouse skin tumours were paraformaldehyde-fixed and paraffin-embedded. Tissue sections (5 μ m) were stained with hematoxylin/eosin. Histological analysis of actinic keratosis and tumour

classification were performed in a blind manner by a pathologist. SCCs were classified according to the Broders' classification based on the degree of SCC keratinization and of keratinocyte differentiation (Broders, 1921). This classification is as follows: SCC Grade I: 75% keratinocytes are well differentiated; SCC Grade II: >50% keratinocytes are well differentiated; SCC Grade III: >25% keratinocytes are well differentiated and SCC Grade 4: <25% keratinocytes are well differentiated. Actinic keratoses were defined histologically and classified as grade I, II or III based on the degree of cytological atypia of epidermal keratinocytes and involvement of adnexal structures according to Rowert-Huber *et al* (Rowert-Huber *et al*, 2007). Representative pictures of actinic keratosis and SCCs are presented in supplementary Fig S12.

Human skin biopsies

Cutaneous SCC samples were obtained anonymously from the Department of Dermatology, University Hospital of Lausanne, Switzerland. Normal skin was from healthy adult volunteers or from the edges of skin tumours. SCC was diagnosed by experienced pathologists. Informed consent for research was obtained prior to routine diagnostic services. All samples include the dermis and the epidermis.

Preparation of expression data

We used expression microarrays from the Expression Project for Oncology Consortium (Affymetrix human genome HG-U133 plus 2.0 arrays) and from the Gene Expression Omnibus (accession code: GSE2109). Only the following tumour types were considered: breast ($n = 344$), lung ($n = 121$), liver ($n = 41$), pancreas ($n = 14$), colon ($n = 277$), ovarian ($n = 202$), prostate ($n = 67$) and kidney ($n = 259$). Other tumour types were excluded because of low sample numbers at the time of download.

Gene expression data were normalized with the robust multi-array method implemented in the library affymetrix. Three selection criteria were applied to probe sets: annotation to a defined Entrez gene, standard deviation of normalized expression greater than 0.1, and characterization as the most variable probe set for a given Entrez gene. The normalized, log-transformed expression measures were used for the analysis. For the OSCC dataset, because two datasets were available from the GEO database, optimal probe set selection among features mapped to TGFB1, SRC and PPARB/D was performed with the GSE30784 dataset and then applied in the GSE41613 (Chen *et al*, 2008; Lohavanichbutr *et al*, 2013). Only results obtained with the GSE41613 were considered in the main analysis. For the SSC dataset, the GSE32628 was taken using the normalized values provided by the contributor (Hameetman *et al*, 2013).

All data were analysed in the R programming environment (2.9.1). Linear regression models were used to assess the relationships between gene expression levels using additive and multiplicative models (interactions). Linear models were applied separately to each tumour type. A meta-analysis of the interaction coefficient estimates for the various tumour types was performed using a random effect model.

Src cDNA isolation, polyA-tailing and 5' RACE

To identify *Src* transcript isoform(s) induced by PPAR β/δ in skin and keratinocytes, *Src* mRNA was specifically reverse transcribed

with Superscript II (Invitrogen) using 50 ng of primer GSP1 (Gene Specific Primer 1, primer sequence given in supplementary Table S2) from 5 μ g of total RNA (extraction with TRIzol, Invitrogen) from a culture of wild-type primary keratinocytes treated for 24 h with GW501516 (100 nM) or total skin from wild-type SKH-1 mice acutely irradiated and sacrificed 24 h later. After treatment with RNase H (New England Biolabs) for 20 min at 37°C, first-strand Src cDNA was purified (Macherey-Nagel) before a polyA-tailing reaction in the presence of Tdt (Promega) and ATP. A first, PCR was performed using Frohmann and GSP2 (Gene Specific Primer 2) primers (Fig 3; sequences given in supplementary Table S2) using Herculanase II Fusion DNA Polymerase (Agilent Technologies) according to the manufacturer's instructions. A nested PCR was then performed with Q0 and GSP3 (Gene Specific Primer 3) primers (Fig 3; sequences given in supplementary Table S2) using Herculanase II Fusion DNA Polymerase (Agilent Technologies). PCR products were then purified (Macherey-Nagel) and loaded onto agarose gels for identification of the Src transcript(s). GSP1, GSP2 and GSP3 primers were designed to specifically amplify a region corresponding to Src-001, Src-007, Src-005, Src-006 or Src-201 transcripts described in Ensembl. The DNA bands revealed were then excised and purified before sequencing.

PPRE subcloning, mutagenesis and promoter activity assay

To analyse Src promoter activity, various regions of the promoter (annotated from the transcriptional start site in the mouse genome) were inserted into the plasmid vector pGL4 (Promega, E8421) to generate pGL4-PPRE1, pGL4-PPRE2, pGL4-PPRE3, pGL4-PPRE4 and pGL4-PPRE5. pGL4-PPRE mutant plasmids carried mutated PPRE sequences introduced with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primers used for mutagenesis are given in supplementary Table S1. NIH3T3 cells were plated the day before transfection in 12-well plates. Either 0.8 μ g of wild-type or mutated pGL4-PPRE or 0.5 μ g of 3 $\times$ -PPRE-TK-Luc (gift from R. Evans) plasmids were introduced into the cells via Superfect (Qiagen), together with 0.5 μ g of pSG5-mouse-PPAR β/δ and 0.025 μ g of a Renilla luciferase construct. After overnight serum starvation, transfected cells were treated for 24 h with GW501516 (100 nM) and then lysed in 1 $\times$ passive lysis buffer and analysed for dual luciferase activity following the manufacturer's instructions (Promega). Relative luciferase activity was calculated as the ratio of firefly to Renilla luciferase activity. Data represent means $\pm$ standard deviations of relative luciferase activities for DMSO performed in triplicate.

Immunoblotting

Cells and tissue samples were lysed in TNE buffer containing 20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 mM Na₃VO₄. After quantification, proteins were separated by SDS-PAGE and subjected to immunoblotting. All primary antibodies were incubated overnight in 1 $\times$ Tris-buffered saline plus 0.1% Tween-20 and 5% bovine serum albumin at a dilution of 1:1000 except where indicated otherwise. Cell Signalling was the source for the following antibodies: Src (2123), phospho-Tyr845 EGFR (6963), EGFR (4267), phospho-Erk1/2 (4370), Erk1/2 (9102), β -Catenin (8480), phospho-Ser675 β -Catenin (4176), Slug (9585) and GAPDH (2118). Anti- β -tubulin and N-Cadherin were purchased from BD Biosciences (556321) and Millipore (04-1126; 1/50 000), respectively.

ChIP and re-ChIP

ChIP experiments were carried out with modifications of the experimental setup described by Tan *et al* (Tan *et al*, 2004). Briefly, chromatin was crosslinked using 0.5% formaldehyde for 10 min at 37°C and sonicated in SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl, pH 8.1) to obtain crosslinked DNA that measured 200–500 bp in length. Approximately 10% of the supernatant was retained as input, while the remaining amount was processed with the ChIP using anti-PPAR β/δ antibody (4 μ g; SC-7197, TransCruz Grade; Santa Cruz), and complexes were pulled-down by Protein A/G (Santa Cruz, CA, USA). Re-ChIP was performed by subsequent probing with anti-p300 antibody (4 μ g; 05-257, Millipore); DNA fragments were reverse crosslinked at 65°C for 6 h. The pre-immune complex served as negative control. The ChIP primer sequences are listed in supplementary Table S3. Downregulation of PPAR β/δ in MK cells was performed using a siRNA construct against mouse *Ppar β/δ* (L-042751-01, Thermo Scientific), as recommended by the manufacturer (Dharmacon, Thermo Scientific). Briefly, MK cells were seeded in 10-cm dishes and transfected with 25 nM of siRNA in DharmaFECT1 transfection reagent (T-2001-03, Thermo Scientific). MK cells transfected with scrambled siRNA were used as controls. The efficiency of KD was tested by real-time PCR 48 h later (supplementary Fig S5E).

Immunohistochemistry and immunofluorescence

Src, Ki67 and Keratin 13 staining was performed on tissue sections (5 μ m) from paraformaldehyde-fixed, paraffin-embedded skin. Briefly, sections were quenched with 3% H₂O₂. Ki67 (Abcam, 15580) staining was performed as described by the manufacturer. For Src and Keratin 13 staining, the antigen-retrieval step was achieved in 0.1 M citrate buffer [pH 6] via microwave. After washing, sections were incubated in normal goat serum 1% for 30 min and probed with Src (Cell Signalling; 2109; 1:250) and Keratin 13 (Abcam; ab92551; 1:100) antibodies overnight at 4°C or at room temperature in blocking buffer, respectively. After washing, sections were incubated for 30 min with anti-rabbit horseradish peroxidase secondary antibody (EnVision+ rabbit-HRP; Dako; K4002). Visualization was performed with diaminobenzidine substrate (Vector Labs) for 14 min before being counterstained with Mayer hematoxylin and mounted. Keratin 10 and Keratin 14 co-staining and N-Cadherin staining were performed as described above with the following modifications. Tissue sections were probed with Keratin 10 (Progen; GP-K10; 1/200), Keratin 14 (Covance; PRB-155P; 1/1000) or N-Cadherin (Millipore; 04-1126; 1/50) overnight at 4°C, washed, and then incubated 30 min at room temperature with Goat anti-guinea-pig A488 (Molecular Probes; A11073; 1/200) and Goat anti-rabbit A568 (Molecular Probes; A21069; 1/1000), for Keratin 10/Keratin 14 co-staining or Goat anti-rabbit A568 (Molecular Probes; A21069; 1/1000) for N-Cadherin staining. After washing, counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI) for 5 min at RT.

Double staining for laminin 332 and pan-Cytokeratin was performed from OTC-embedded frozen skin samples. Briefly, sections were defrosted and incubated in acetone for 10 min, washed and blocked for 1 h in 1% normal goat serum/5% bovine serum albumin. Sections were probed overnight at 4°C with anti-laminin 332 (Abcam; 14509; 1:200) and anti-pan-Cytokeratin (BMA-T1341; 1:250) in blocking buffer. After being washed, sections were

incubated for 30 min with anti-mouse STAV-Cy3 (Zymed; 43-4315; 1:1800) and anti-rabbit A488 (Molecular Probes; A111034, 1:2000) diluted in blocking buffer. Sections were then washed three times and incubated 5 min with DAPI (Sigma; D95542; 1:5000) and washed again before being mounted.

PLA and immunofluorescence

PLA experiments were performed in accordance with the manufacturer's protocol (Olink Biosciences, Sweden) using pairs of antibodies targeting the protein–protein interactions of interest. The antibodies were laminin 332 (1:20; Abcam, ab14509), active integrin $\beta 4$ (Abcam, ab29042; 1/50) and Rac1 (Abcam, ab97732; 1/20). PLA images were taken using the LSM 710 confocal microscope (Carl Zeiss, Germany) with the Plan-Apochromat 40 $\times$ /1.4 oil differential interference contrast objective. Analyses were performed with the ZEN 2009 Light Edition software (Carl Zeiss, Germany). Quantification of PLA signals was performed with BlobFinder version 3.2 (Allalou & Wahlby, 2009). Immunofluorescence for laminin 332 (1/250) was performed after PLA for integrin $\beta 4$ and Rac1 as previously described.

Epidermis and dermis separation for RNA extraction

Epidermis and dermis were prepared from whole dorsal skin of acutely irradiated mice according to the protocol developed by Clemmensen *et al* (Clemmensen *et al*, 2009), with some modifications. After dorsal skin harvest, adipose tissue was removed with a scalpel on ice, and the skin was cut into small pieces (1–2 mm) and immediately incubated for 15 min at RT in ammonium thiocyanate 3.8% in 1 $\times$ phosphate-buffered saline. Epidermis and dermis were then separated mechanically with forceps in TRIzol. After RNA extraction and quality control by Bioanalyser, RT-PCR and qPCR were performed as described below to generate cDNA. Specific markers of the epidermis (Keratins 10 and 14) and dermis (Collagen 4 $\alpha 1$) compartments were tested by qRT-PCR to check the proper separation of the two in each experiment (supplementary Fig S2D).

Statistics summary

Unless indicated otherwise, all data are presented as the means $\pm$ standard errors of the mean, and statistical differences were evaluated by two-tailed Student's *t*-tests. For all analyses, we considered *p* < 0.05 to be statistically significant.

Study approval

Collection of human skin biopsies was approved by the local (Centre Hospitalier Universitaire Vaudois) and cantonal (Canton de Vaud) research ethics committees. All experiments involving animals were approved by the Veterinary Office of the Canton Vaud (Switzerland) in accordance with the Federal Swiss Veterinary Office Guidelines. All experiments conform to European Commission Directive 86/609/EEC.

Supplementary information for this article is available online: <http://embomolmed.embopress.org>

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The paper explained

Problem

The incidence of non-melanoma skin cancer (NMSC) is increasing at a high rate, with over one million estimated new cases each year in the United States alone. Squamous cell carcinoma (SCC) and basal cell carcinoma, which both derive from keratinocytes, account for approximately 96% of all diagnosed skin cancers. Although mortality from NMSC is low, morbidity is high and is accompanied by a heavy personal burden and enormous rising costs for society. NMSCs are associated with excessive and/or chronic exposure to ultraviolet (UV) radiation, especially UVB, and mainly occur on sun-exposed areas. Despite the prevalence of NMSCs, the molecular mechanisms underlying their progression remain poorly defined. In skin, roles for the peroxisome proliferator-activated receptor beta/delta (PPAR β/δ), a member of the nuclear receptor superfamily, have been emphasized by its implication in keratinocyte adhesion, migration and survival during tissue repair. These processes also participate in skin cancer progression. However, whether PPAR β/δ is pro- or anti-carcinogenic remains controversial because both effects have been observed. We therefore investigated *in vivo* whether PPAR β/δ might be involved in UV-induced skin carcinoma, using SKH-1 hairless albino mice lacking PPAR β/δ activity, a relevant model for studying human UV-induced skin cancer.

Results

In vivo and *in cellulo*, we characterized a cascade of events involving PPAR β/δ as a regulator of oncogene *Src* transcription. We found that UV induces PPAR β/δ activity, which in turn stimulates *Src* gene expression, resulting in increased *Src* protein expression. We further demonstrated that UV exposure activates *Src* and enhances the EGFR/Erk1/2 signalling pathway, resulting in increased PPAR β/δ -dependent pro-tumoural gene expression. In line with this, PPAR β/δ -null mice developed fewer and less advanced tumours compared to wild-type mice. Moreover, topical application of a PPAR β/δ antagonist prevented UV-dependent stimulation of the *Src*/EGFR/Erk1/2 signalling pathway. In human SCCs, *PPARB/D* expression was also positively correlated with *SRC* expression. In a wide variety of human carcinomas from various tissue origins, we identified an interaction among *PPARB/D*, *SRC* and *TGFB1* at the transcriptional level, which suggests a broad pro-tumourigenic potential of PPAR β/δ via *SRC* upregulation.

Impact

Our present work motivates the future evaluation of PPAR β/δ antagonists as putative anti-cancer drugs that could be used to prevent skin carcinoma progression and, potentially, epithelial cancers from other tissue origin. Interestingly, Desatinib (Sprycel), a dual BCR/ABL and *Src* family tyrosine kinase inhibitor previously used in patients with acute myeloid leukaemia, is currently being assessed in a phase II clinical trial for its ability to inhibit *Src* activity and treat SCC. The identification of PPAR β/δ as a regulator of *Src* gene expression will further help to refine this therapeutic strategy.

In addition, interest is increasing in PPAR β/δ agonists for treating patients with metabolic syndrome through a stimulatory effect on lipid oxidation in skeletal muscle. A PPAR β/δ agonist is currently being tested in a phase IV clinical trial with obese patients showing dyslipidemia. Our data underscore the necessity of a careful evaluation of the use of high-affinity PPAR β/δ agonists, especially in conditions necessitating long-term treatment.

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Author Contributions

AM and MBD designed and performed experiments and analysed the data. CTB, HM, JSKC, MKS, CM, GD, MA and MB performed experiments. TAL, YL, SW and NST provided material and were involved in manuscript preparation. DH and TAS screened and graded mouse tumours. Biostatistics analysis of human tumour mRNA expression profiles was conducted by PJF. LM and WW supervised the project, designed experiments and analysed the data. This manuscript was written by AM, LM and WW. All authors reviewed the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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